ANTIPSYCHOTIC THERAPEUTIC DRUG MONITORING EVALUATION OF THE ROLE OF ORAL FLUID, AND PLASMA QUETIAPINE METABOLITE ASSAYS

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ANTIPSYCHOTIC THERAPEUTIC DRUG MONITORING: EVALUATION OF THE ROLE OF ORAL FLUID, AND PLASMA QUETIAPINE METABOLITE ASSAYS

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Abstract

Therapeutic drug monitoring (TDM) is the measurement of drugs and/or metabolites in biological samples to guide therapy. TDM for antipsychotic drug therapy in schizophrenia treatment is well-established if adherence is in doubt and in dose optimisation.

The antipsychotic quetiapine has a short plasma half-life and no clear reference range for clinical response. Three quetiapine metabolites, *N*-desalkylquetiapine, *O*-desalkylquetiapine and 7-hydroxyquetiapine, were investigated in routine plasma TDM samples to determine whether they provide useful information to assess quetiapine exposure. Compared to median plasma quetiapine concentrations, *N*-desalkylquetiapine, *O*-desalkylquetiapine and 7-hydroxy-quetiapine plasma concentrations were equivalent, $1/10^{th}$ and $1/20^{th}$, respectively. All metabolites showed greater correlation to dose than quetiapine itself; dose-related plasma concentrations have been presented to aid future results interpretation.

Blood sampling is invasive; hence, oral fluid was investigated as an alternative matrix. A clinical study was undertaken to investigate the relationship between analyte concentrations in oral fluid samples collected using different oral fluid collection devices [Greiner Bio-One (GBO; in-mouth buffered collected system), and Thermo Oral-Eze] and in plasma. Existing plasma LC-M/MS methods were combined, and sample preparation modified to be suitable for acidified (buffered) oral fluid samples. The methods were assessed to ensure they were fit-for-purpose.

Relationships between plasma and oral fluid concentrations of clozapine and norclozapine were poor, and no better than results obtained using unstimulated oral fluid collected via the drool method. Median concentrations of all analytes were lower in the GBO samples than Oral-Eze samples; however, clozapine and norclozapine concentrations in samples collected using the proprietary devices were approximately twice the concentration measured in samples collected by the drool method, as a ratio to plasma concentrations. Concentration differences are possibly related to differing salivary stimulation during sample collection. A positive finding was a patient who had become non-adherent to clozapine was identified from oral fluid as well as plasma analysis.

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Glossary and abbreviations

Term	Meaning
Agranulocytosis	Deficiency of white blood cells in the blood
APCI	Atmospheric pressure chemical ionisation
C	Plasma analyte concentration
C/D	Dose-corrected plasma analyte concentration
CI	Confidence intervals
CYP	Cytochrome P450
D	Deuterium
Drool	Oral fluid collected by pooling in the mouth then spitting into a container
ER	Extended or modified release drug formulation
EPS	Extra-pyramidal side effects
ESI	Electrospray ionisation
FGA	First generation ('typical') antipsychotic drug
FIA	Flow injection analysis
GBO	Greiner Bio-One oral fluid collection device
GI	Gastro-intestinal
IR	Immediate release drug formulation
IS	Internal standard
IQC	Internal quality control solution
LC	Liquid chromatography
LLE	Liquid-liquid extraction
m/z	Mass to charge ratio
MR	Metabolic ratio (concentration of metabolite / concentration of parent)
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Norclozapine	*N*-Desmethylclozapine
Norfluoxetine	*N*-Desmethylfluoxetine
OF	Oral Fluid
Oral fluid %	Content of oral fluid present in the GBO collected fluid as a percentage
Oral-Eze	Oral-Eze oral fluid collection device
PI	Prediction intervals
RP	Reversed phase chromatography
RSD	Relative standard deviation
SCX	Strong-cation exchange
SD	Standard deviation
SGA	Second generation ('atypical') antipsychotic drug
STD	Calibration solution
TDM	Therapeutic drug monitoring
Tris	*Tris*(hydroxymethyl)aminomethane
TRS	Treatment resistant schizophrenia
TSLD	Time since last dose
U/mL	Saliva content in units per mL of collection fluid
UV	Ultra-violet detection

Chapter 1. Introduction

1.1 Therapeutic drug monitoring

During the course of routine drug therapy, the aim is to ensure that the correct amount of drug is present within the patient's system to provide a beneficial outcome and reduce the risk of adverse drug reactions. Optimising the treatment regime to the individual can ensure the drug is given a fair trial, rather than the treatment being altered unnecessarily in cases where the dose was not suitable. For many drugs there are established doses at which a clinical response is predicted and where toxicity is unlikely, such as in the case of paracetamol where taking a standard dose predicts therapeutic benefit. However, for some drugs there is a poor correlation between administered dose and therapeutic benefit. Often this poor correlation is related to the mode of administration and distribution to the active site; for example, in muscle pain, topical pain relief applied directly to the site of pain may be fastest way to reduce pain in that area, but this would not be an appropriate form of administration for drugs that act on other organs. Drugs that are active in the brain, for example, must usually circulate in the blood and then distribute across the blood-brain barrier before reaching the site of activity and therefore there are many factors that can lead to large between-patient variation in response at a given dose.

Factors that can affect how much of an oral dose is present in the bloodstream and thus circulates within the body include oral bioavailability (i.e. the proportion of the administered dose that enters the systemic circulation), the rate of liberation of the drug from the formulation, and the plasma half-life of the drug, which is affected by the individual's metabolic capacity for the drug in question. Adherence to the treatment regime is also an important factor with oral therapy and may manifest as partial adherence where some of the prescribed dosage is taken, but not all or not consistently, or total non-adherence where the patient does not take any of the prescribed medication for a period of time.

Therapeutic drug monitoring (TDM) is a method by which the concentration of a drug is measured within a clinical sample, often whole blood or plasma. For TDM to be of clinical value there must be a correlation between the clinical effect and the concentration of the drug and any active metabolite(s) in the relevant matrix, i.e. a reference range for therapeutic benefit, as well as a concentration above which there is an increased risk of toxicity. TDM is therefore used to assess adherence, establish dose adequacy and minimise the risk of toxicity.

TDM is used within many fields, one of which is psychiatry. Often in this field medication is essential and can provide many benefits, both medical and economic, when the therapy is successful (Hiemke *et al*., 2011). The main requirement for TDM within this field is due to the sometimes 20-fold variation in dose required to attain equivalent plasma concentrations between patients, so that after changes in co-prescribed medications, or taking into account pharmacogenetics factors, a suitable dose can be administered to each patient to achieve the highest possible chance of response with the lowest risk of toxicity (Hiemke *et al*., 2011).

Drugs in the systemic circulation are commonly bound to plasma proteins, such as albumin or alpha-1 acid glycoprotein, depending on the physiochemical properties of the compound in question. There are believed to be a finite number of plasma protein binding sites, and therefore analytes can be displaced or compete for active sites, meaning the amount of analyte bound to plasma proteins can differ for the same drug in different individuals, or in the same individual following changes to co-prescribed medications (Patsalos and Berry, 2013); disease states such as acute kidney failure can also alter circulating protein concentrations (Patsalos and Berry, 2013).

Since the site of activity of centrally-acting drugs is through the blood-brain barrier, the portion of drug that is free (unbound) in plasma is that which is able to cross this membrane and hence this may sometimes be classified as the ‘active’ fraction. In general TDM undertaken on plasma will quantify the total, rather than free, fraction and hence the concentrations may not always be truly representative of activity if the concentrations of binding proteins or binding sites change between samples. Quantifying the free fraction requires relatively complex sample preparation techniques, which are often more expensive and therefore less utilised within routine TDM than measuring total drug concentrations. In addition, the assay methods used are likely to alter the free fraction leading to potentially misleading results.

Alternative means to estimate the free fraction can include the use of oral fluid (OF). Oral fluid is a matrix formed mainly from saliva, the largely water-based fluid secreted by the salivary glands, but also includes proteins (including enzymes, albumin and mucin), electrolytes and cell debris. The portion of drug that enters the salivary ducts and is secreted into oral fluid has been claimed to represent the free fraction for some drugs including anticonvulsant drugs (Patsalos and Berry, 2013). Another advantage of oral fluid is that collection is non-invasive compared to plasma samples, since collection does not require venepuncture.

1.2 Antipsychotic drugs

Antipsychotic drugs (also known as neuroleptic drugs) are the mainstay of the treatment of schizophrenia, a disease that affects some 2 % of the population. Schizophrenia generally arises in early adulthood, with the onset usually being earlier and with greater severity and higher prevalence in males compared to females (Flanagan, 2006). Overall the symptoms can be divided into three broad categories: positive symptoms, which include delusions, hallucinations, and impaired communication; negative symptoms such as social withdrawal and lack of drive; and cognitive dysfunction, which affects insight, memory and reasoning.

First generation ('typical') antipsychotics (FGAs), such as chlorpromazine, that were introduced in the early 1950s, were found to relieve some of the positive symptoms of schizophrenia including hallucinations, delusions and hyperactivity (Flanagan, 2006). However, these medications are generally not as effective against negative or cognitive symptoms and can cause a high prevalence of side effects especially those known as extra-pyramidal side effects (EPS; Taylor *et al.*, 2015). EPS are repetitive involuntary movements of muscles or limbs similar to those seen in Parkinson disease, and can progress to tardive dyskinesia where the involuntary movements become more pronounced and can persist even after discontinuation of the medication, causing lifelong disability.

Development of second generation ('atypical') antipsychotics (SGAs) such as clozapine gave drugs that were effective against both the positive and negative symptoms as well as improving cognitive impairment through much broader neurotransmitter antagonism (Flanagan, 2006). These drugs generally display lower risk of EPS than FGAs, however they have been associated with an increased risk of raised plasma prolactin, other biochemical abnormalities, sexual dysfunction and weight gain (Taylor *et al.*, 2015). Newer antipsychotics, such as aripiprazole, have been developed to act as both antagonists and partial agonists on neurotransmitter activity, and some evidence suggests aripiprazole is as effective as other SGAs whilst causing fewer side effects (Taylor *et al.*, 2015).

Treatment-resistant schizophrenia (TRS) is the term used to denote a form of the illness where at least two antipsychotics have been trialled without benefit; clozapine is the only antipsychotic drug that has proven benefit in these cases (Taylor, 2017). Due to the nature of schizophrenia, adherence to antipsychotic drug medication regimes can be low. The role of TDM in guiding treatment with SGAs is well-established if adherence is in doubt and in dose optimisation,

especially for clozapine, and may be useful in dose adjustment with quetiapine, amisulpride and olanzapine (Hiemke *et al*., 2011).

Antipsychotic drugs are commonly prescribed for oral use, either as a normal (immediate) release tablet (IR), an orodispersible medication, or as an extended/modified release (ER) formulation. In ER formulations a coating is applied that is resistant to digestion/dissolution in the gastrointestinal tract and therefore the drug is liberated more slowly than in normal release formulations, with the aim of giving an extended duration of drug action. There are also some medications that are available as an intra-muscular injection, which can be given weekly, 2-weekly, or monthly, and release the medication slowly into the systemic circulation. Commonly, such formulations are used in patients that are poorly adherent to a daily or bi-daily oral treatment regime.

In general SGAs are basic lipophilic drugs, as this enables them to cross the blood-brain barrier to the site of activity, and often they are significantly plasma protein bound (Table 1.1), although there are exceptions, most notably amisulpride and sulpiride, which are much more water soluble and less protein bound in plasma than other antipsychotics, and are largely excreted unchanged.

Table 1.1: Physiochemical properties of analytes of interest
* complied from Baselt (2014); # compiled from Flanagan (2006)

	Plasma half-life (h) *	pKa (base) *	LogP #	Fraction bound to plasma protein*	Major plasma metabolites
Amisulpride	11 - 27	9.4	1.10	0.17	Nil
Aripiprazole	60 - 90	7.6	4.70	0.99	Dehydroaripiprazole
Clozapine	6 - 17	7.6	3.23	0.95	*N*-Desmethylclozapine (Norclozapine)
Fluoxetine	24 - 72	9.5	4.05	0.94	*N*-Desmethylfluoxetine (Norfluoxetine)
Olanzapine	21 - 54	7.4	1.7 *(pH7)*	0.93	Nil
Quetiapine	2.7 - 9.3	6.8	4.31	0.83	*N*-Desalkylquetiapine
Risperidone	2 - 20	8.2	3.49	0.90	9-Hydroxyrisperidone
Sulpiride	4 - 11	9.0	0.57	0.40	Nil

1.2.1 Clozapine

Clozapine is the only drug with proven efficacy in TRS, but is associated with significant toxicity in normal use. Clozapine was initially developed in the 1960s, but some 2 % of patients suffered from unexplained agranulocytosis (life-threatening reduction in white blood cells), and hence the drug was withdrawn from clinical use. No drug has yet been developed that has shown the success of clozapine in TRS, and therefore in the 1990s it was relicensed under strict provisions that all patients undergo routine haematological monitoring to identify cases of impending agranulocytosis. Agranulocytosis generally presents within the first few months of therapy, hence new patients are monitored weekly for the first few months, with the regularity decreasing to monthly for the remainder of therapy as the risk of developing agranulocytosis stabilises (Bleakley and Taylor, 2013).

Clozapine is very toxic in someone who has either not taken it before, or who has lost tolerance to the drug; a routine daily dose of clozapine can kill a clozapine-naïve patient or someone who has not taken the medication for a few days (Bleakley and Taylor, 2013), hence therapy is initiated via cautious dose titration. Clozapine can rarely cause myocarditis and cardiomyopathy, possibly due to direct toxicity to the heart tissue or through an immune response, which may prove fatal if not diagnosed in life (Khan *et al.*, 2017). On the other hand, gastrointestinal (GI) toxicity is very common during clozapine therapy, generally in the form of GI hypomotility (e.g. constipation) that if not monitored and treated can lead to severe symptoms, including faecal vomiting and GI rupture, which can prove fatal (Bleakley and Taylor, 2013). Other side effects include an increased risk of convulsions at higher doses or plasma concentrations, hypersalivation, and biochemical abnormalities, as mentioned above.

Clozapine is metabolised primarily to an *N*-demethylated product *N*-desmethylclozapine (norclozapine) via cytochrome P450 (CYP) 1A2, CYP 3A4 and CYP 2D6. CYP 2D6 and CYP 3A4 have a number of genetic polymorphisms that cause wide between-patient variability in enzyme activity. The activity of CYP 1A2 also displays variability between patients, and is affected by a number of factors including cigarette (and cannabis) smoking and co-prescribed medications. Polycyclic aromatic hydrocarbons inhaled when smoking induce the activity of CYP 1A2, meaning that patients who smoke require a higher dose on average of clozapine than patients who do not smoke, and stopping smoking can cause the plasma clozapine concentration to increase dramatically unless the dose is cut. The antidepressant fluvoxamine

and antibiotic ciprofloxacin, as well as some other drugs, significantly inhibit CYP 1A2 activity and therefore addition of these drugs can cause clozapine plasma concentrations to increase leading to toxicity at a constant clozapine dose. Overall, variation of clozapine concentrations attained between different individuals for a given dose can vary up to 50-fold (Couchman *et al*., 2010). As such, attaining the optimal therapeutic dose for individual patients can be very challenging; the use of TDM is therefore well established for this drug, especially during initial titration, at a change in dose or co-prescribed medication, and when the patient stops or starts smoking (Hiemke *et al*., 2011).

1.2.2 Quetiapine

Quetiapine is an SGA that is predominantly used in the treatment of schizophrenia and bipolar disorder (mania and depression); also, the ER formulation is used as an adjunct in patients with major depressive disorder in cases where antidepressant monotherapy has had a sub-optimal response (Al Jurdi *et al.*, 2010; Anderson *et al.*, 2009; El-Khalili *et al*., 2010; Ketter *et al.*, 2016: Liebowitz *et al.*, 2010; Mauri *et al.*, 2016; McIntyre *et al.*, 2009). Whilst not licensed for the indications, quetiapine is increasingly being shown to be effective in the treatment of anxiety and sleep disorders (Altamura *et al.*, 2011; Baune *et al*., 2007; Kreys and Phan, 2015; Maneeton *et al.*, 2016; Stein *et al*., 2011).

Broadly, quetiapine is well tolerated and patients do not describe significant toxicity. However, quetiapine is linked to a risk of increased body weight and raised metabolic markers including plasma triglycerides with increasing prescribed dose (Zhornitsky *et al*., 2011). In overdose, plasma quetiapine concentrations were reported to be greater than 3000 µg/L, approximately 10-times those suggested for therapeutic benefit, with reported symptoms of only tachycardia and somnolence (Hunfield *et al.*, 2006).

Quetiapine is metabolised mainly by CYP 3A4, with a contribution from both CYP 2D6 and CYP 3A5 (DeVane and Nemeroff, 2001), forming a number of metabolites. *N*-Desalkyl-quetiapine (widely referred to as norquetiapine) is the major metabolite believed to be pharmacologically active in the treatment of depression and may mediate the antidepressant effect of quetiapine at least in part due to greater activity at receptor sites than the parent compound (Di Benedetto *et al.*, 2012; Jensen *et al*., 2008; López-Muñoz and Alamo, 2013). 7-Hydroxyquetiapine and 7-hydroxy-*N*-desalkylquetiapine may possess antipsychotic activity; however, they accumulate to much lower concentrations in plasma than quetiapine itself (Davis

et al., 2010; Fisher *et al*., 2012A; Li *et al.*, 2004). There is a lack of data as to the plasma concentrations of quetiapine sulfoxide and quetiapine carboxylic acid attained during therapy, and neither are thought to display any pharmacological activity, although these compounds have been found in urine (Strickland *et al.*, 2016; Winter *et al.*, 2008).

Quetiapine has a plasma half-life of around 7 hours, reaching maximum concentrations around 1 hour after an oral dose (Figueroa *et al.*, 2009), meaning twice-daily dosing is often required to maintain steady state plasma concentrations over the course of a day. The ER formulation aims to release quetiapine over the course of 20 hours with maximal concentrations at 5 hours after an oral dose, reaching lower maximal concentration, but giving equivalent overall exposure compared to the IR formulation (Bui *et al*., 2013; Figueroa *et al*., 2009). As such, ER quetiapine can be given once-daily, at night, which may reduce daytime somnolence (Riedel *et al.*, 2015). The plasma half-life is believed to be around 8 hours for 7-hydroxyquetiapine and 7-hydroxy-*N*-desalkylquetiapine (Li *et al.*, 2004), with *N*-desalkylquetiapine around 11 hours (Winter *et al.*, 2008). Due to the longer half-life of *N*-desalkylquetiapine, compared to quetiapine and the other metabolites, plasma concentrations are less affected by formulation or time that the sample was taken after dose, suggesting this could be a better marker than plasma quetiapine or other metabolite concentrations for assessing quetiapine exposure than measuring quetiapine itself (Fisher *et al*., 2012A).

1.2.3 Other relevant drugs

Olanzapine, amisulpride, risperidone and Paliperidone (±-9-hydroxyrisperidone) are SGAs for which plasma TDM is offered, and reference ranges associated with effective therapy have been suggested for these drugs (Hiemke *et al*., 2011). In addition, aripiprazole and sulpiride (commonly referred to as an FGA) are routinely analysed in plasma, although TDM is generally used only to assess adherence since a reference range for effective therapy is not firmly established and side effects are less severe.

Finally, the antidepressant fluoxetine (Prozac) was included within the study due to the large number of prescriptions of this medication in children within the local mental health hospital, and concerns over adherence in this population (Nakonezny *et al.*, 2010).

1.3 Methodology

In order to get meaningful TDM results there must be a high degree of confidence that the reported result is indicative of the circulating drug or metabolite concentrations. In order to achieve this, the methodology that is used to quantify the analyte(s) in question must be shown to be fit-for-purpose; that it is sensitive, selective and accurate enough for the concentrations to be measured in the samples available (FDA/CDER, 2001). There are a number of possible steps involved in the quantification of analytes from a given matrix, and modifying the application of each step enables development of a method that is more or less selective and sensitive depending on individual need.

Sample preparation is the means by which analytes within a sample are prepared for detection and quantification. Sample preparation can range from simple protein-precipitation, which can lead to a number of interferences being present during an analysis, to more complex forms of sample clean-up such as solid-phase extraction and liquid-liquid extraction (LLE) that are more labour-intensive, but usually lead to cleaner extracts and therefore more specificity and selectivity. LLE can be used to make an analytical method very selective since the choice of extraction solvent and pH can be optimised to the analyte(s) of interest in order to exclude interferences, and when coupled to strong-cation exchange (SCX) chromatography the selectivity and robustness of methods for basic (proton-accepting) analytes can be enhanced (Flanagan *et al*., 2001).

Chromatography is commonly used to separate analytes within an extract in order improve selectivity and hence, minimise interferences during the analysis. Separation is typically undertaken by the use of a stationary phase within a column that contains an active group that binds analytes flowing through the column following injection into the mobile phase. Binding affinity depends on the analyte in question as well as the selection of mobile phase and stationary phase, where modifying each parameter can affect the separation of the analyte from any interferences present in the extract. The selection of mobile phase is also determined by the type of detection that is selected; most commonly detection is by mass-spectrometry, where the mobile phase flows into a source that ionises analytes within the column effluent and uses mass and ionisation as a means of investigating the content of the flow at a point in time. A number of methods for quantifying SGA concentrations attained during therapy have been published, many of which use liquid chromatography (LC) with mass spectrometry (MS) (Patteet

et al., 2015). Use of tandem mass spectrometry (MS/MS), in which a precursor ion of specified mass-to-charge ratio (*m/z*) is fragmented under defined conditions to give product ion(s) of different *m/z*, can provide additional selectivity and hence sensitivity. Addition of a stable isotope-labelled internal standard (IS) to the sample prior to extraction helps compensate for extract-to-extract variations during sample preparation and analysis. Optimising the sample preparation, separation and detection can enable faster analysis times to aid sample throughput, thereby ensuring the results are available as soon as possible to the clinical team.

Application of method selection techniques has enabled development of a selective method capable of analysing a number of SGAs, as well as aripiprazole and sulpiride, and their major plasma metabolites in a single plasma sample (Fisher *et al*., 2013B), and has been modified to include three additional quetiapine metabolites (*N*-desalkylquetiapine, *O*-desalkylquetiapine and 7-hydroxyquetiapine; Fisher *et al*., 2012B).

1.4 Alternative Samples

Plasma is the traditional sample type for antipsychotic TDM purposes, and such measurements are the basis of most reference ranges (Hiemke *et al*., 2011). However, obtaining venous blood is an invasive procedure. Patients may be unwilling to provide samples or there may be difficulty obtaining such a sample, for example if a vein collapses, and there are additional considerations in the case of children. Collection of a blood sample is ideally only undertaken if there is no diagnostic alternative, and in the case of clozapine the requirement for regular haematological monitoring is a common reason that patients refuse treatment with the drug (Gee *et al*., 2014). However, advances in haematological monitoring using capillary (finger-prick) samples may in the future minimise the requirement for venepuncture in clozapine patients.

Alternative matrices to plasma, such as oral fluid and capillary blood, enable a sample to be taken from patients less invasively than by venepuncture. However, prior to implementation a correlation must be shown between analyte concentrations in the matrix of choice and clinical response, often established through a correlation to plasma concentrations (Fisher *et al*., 2013A; Patsalos and Berry, 2013; Goosen *et al*., 2003). Oral fluid has been well proven as a matrix of choice for TDM of some (acidic) drugs such as anticonvulsants (Patsalos and Berry, 2013), but there is little or no evidence that implementation of alternative matrices for antipsychotic TDM is feasible (Langman *et al*., 2007; Patteet *et al*., 2016).

Previous work using the drool method (unstimulated pooling of oral fluid in the mouth and spit into a tube) to collect samples from patients prescribed SGAs showed that there was a relationship between plasma and oral fluid concentrations of amisulpride, clozapine, quetiapine and risperidone (Fisher *et al*., 2013A). However, the correlations to plasma concentrations were low (R^2 = 0.3-0.7), suggesting that whilst oral fluid analysis is feasible, the possibility of using oral fluid collected by this method to replace plasma for antipsychotic TDM was remote.

One possible reason for the poor correlations between plasma and oral fluid concentrations in this latter study was the use of unstimulated oral fluid, which may be susceptible to between-patient and between-collection variations in salivary pH and flow rate, variables that are thought to affect oral fluid concentrations of basic drugs (Aps and Martens, 2005; Drummer, 2008; Patsalos and Berry, 2013). Investigation of stimulated collection methods, including one that utilises a buffer held in the mouth to reduce variation in oral cavity pH during sample collections, may minimise the variability in the plasma:oral fluid relationship and thus facilitate use of oral fluid for TDM of basic drugs such as SGAs.

1.5 Aims for the thesis

Firstly, this work aims to extend previous work (Fisher *et al*., 2013A) investigating whether measuring three quetiapine metabolites (*N*-desalkylquetiapine, *O*-desalkylquetiapine and 7-hydroxyquetiapine) enhances the value of quetiapine TDM. This will be in the form of an audit of reported analyte concentrations from plasma samples sent to the Toxicology Unit, King's College Hospital (Viapath), for routine quetiapine TDM since September 2009, extending the dataset further than 2012 when the previous audit was completed.

Secondly, the use of two commercial oral fluid collection devices will be investigated for applicability in antipsychotic TDM. The devices of interest are the Greiner Bio-One (GBO) collection device that uses an in-mouth buffered solution, and the Thermo Oral-Eze collection device that uses an adsorbent collection pad held against the cheek.

Plasma LLE-LC-MS/MS methods (Fisher *et al*., 2013B; Fisher *et al*., 2012B) will be combined to allow analysis of all relevant analytes a single sample, including clozapine and norclozapine, and fluoxetine and *N*-desmethylfluoxetine (norfluoxetine). Once the detection has been optimised the sample preparation step will be investigated in order to develop an analytical

method to quantify the relevant analytes in the collection fluid from the two oral fluid collection devices.

Once developed, the methods will be assessed to ensure they are fit-for-purpose.

Finally, the methods will be used to quantify the analyte concentrations in samples collected using the oral fluid devices and in plasma from patients prescribed clozapine, following application to and achievement of ethics approval (Appendix A). Full sample sets will be collected from patients, first the Oral-Eze sample, followed by the GBO sample, and then blood will be collected to separate and store plasma. The analyte concentrations in each matrix from the sample sets will be quantified, patient demographics and dosage information collated, and the results will be studied to establish if samples collected using either of these devices display adequate predictive capacity to replace plasma analysis for TDM of clozapine and other basic drugs.

Chapter 2. TDM of quetiapine metabolites

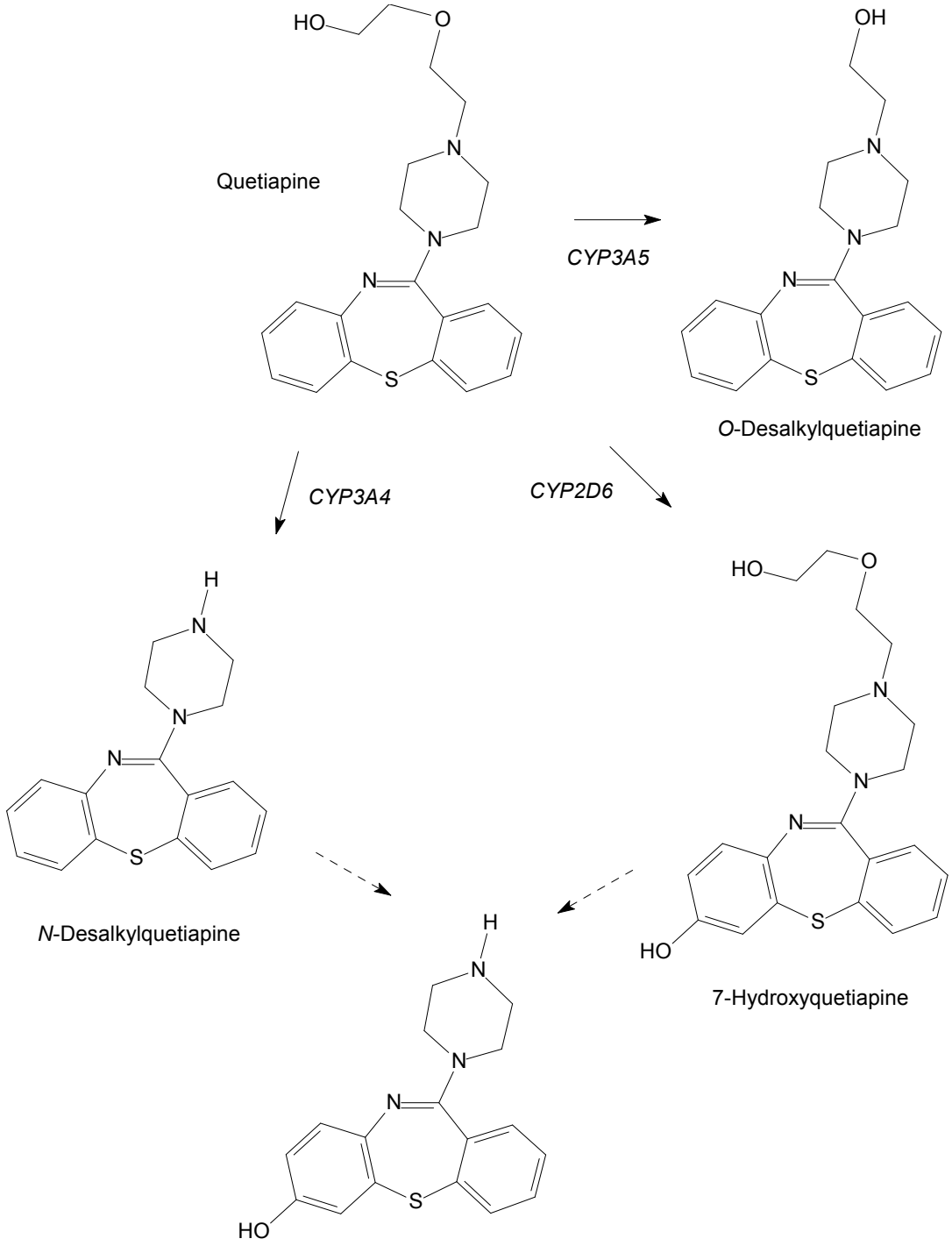
2.1 Introduction

TDM for quetiapine is recommended in patients that are not responding to the medication, or if there are changes to co-prescribed medication (Hiemke *et al.*, 2011), especially when adding or removing drugs that may alter quetiapine plasma concentrations (e.g.. CYP 3A4 inducers, such as carbamazepine). There is no firm reference range for quetiapine, at least in part due to the large variability in quetiapine plasma concentrations observed in therapy (Sparshatt *et al*., 2011). Quetiapine exposure varies widely between patients; in one study quetiapine exposure (presented as dose-corrected plasma quetiapine concentration) varied 238-fold between samples (Hasselstrøm and Linnet, 2004). One possible explanation for this is between-patient variability in CYP activity combined with possible effects of age, sex, and/or co-prescribed medication on quetiapine metabolism. Co-prescription of drugs that affect the activity of relevant CYPs and of proteins such as P-glycoprotein (PGP) that influence drug transport across membranes, such as inhibitors, inducers or competitive substrates, may affect the plasma quetiapine concentration at a constant quetiapine dose.

Whilst there are some published reports on the effect of variables such as age, sex and co-prescribed medication on the plasma quetiapine concentrations attained during therapy (Aicchorn *et al.*, 2006; Bakken *et al*., 2011; Castberg *et al.*, 2007; Handley *et al*., 2013; Hasselstrøm and Linnet, 2004; Isbister *et al.*, 2007; Ostad Haji *et al.*, 2013; Wittmann *et al.*, 2010; Wong *et al*, 2001) little information is available regarding the relevance and predicted concentrations of other quetiapine metabolites that are known to accumulate in plasma, especially for *O*-desalkylquetiapine and 7-hydroxyquetiapine (Figure 2-1).

Preliminary work was undertaken to investigate the importance of the metabolites in interpreting quetiapine exposure (Fisher *et al*., 2012A), but this work included only a small cohort of patient samples leading to difficulty in establishing the significance of the variables studied. Measurement of quetiapine metabolites, especially *N*-desalkylquetiapine with its longer plasma half-life, may enable reference ranges for these compounds to be established thus giving greater insight into the clinical picture.

Figure 2-1: Summary of quetiapine metabolism
Cytochrome P450 (CYP) enzyme believed to play a major role in the formation of each metabolite given, and proposed formation routes indicated by arrows. Compiled from Bakken *et al.*, 2009 and DeVane and Nemeroff, 2001



2.1.1 Aims of the chapter

The results and patient demographics from samples submitted for routine quetiapine TDM at the Toxicology Unit, King's College Hospital (Viapath), 2009-2016 will be compiled and anonymised. Using this dataset, correlation and regression studies will be undertaken to investigate whether quantification of quetiapine metabolites might provide additional knowledge to aid clinical interpretation in quetiapine TDM.

2.2 Materials and methods

The results from plasma samples sent for routine plasma quetiapine TDM at the Toxicology Unit, King's College Hospital (Viapath), from September 2009 to October 2016 were compiled from the clinical database, anonymised, and subjected to audit. Overdose samples, post-mortem samples, or those querying plasma quetiapine concentrations in patients who were not prescribed the medication were excluded. Information given at the time of submitting samples for analysis was collated, and included age and sex of the patient, time and date of sample, time since last quetiapine dose (TSLD, h), prescribed quetiapine dose (mg/d), and formulation [IR or ER].

2.2.1 Analytical method

Analysis was undertaken within the Toxicology Unit, King's College Hospital (Viapath) by LC-MS-MS (Fisher *et al.,* 2012B), and results were reported according to normal clinical practices. Plasma quetiapine, *N*-desalkylquetiapine, *O*-desalkylquetiapine and 7-hydroxy-quetiapine were reported to the nearest 1 µg/L. Lower limits of quantitation were: quetiapine and *N*-desalkylquetiapine, 5 µg/L; *O*-desalkylquetiapine and 7-hydroxyquetiapine, 2 µg/L.

2.2.2 Statistical methods

Statistical analysis was undertaken using Excel Analyse-It (v2.30 Excel 12+) and SPSS (Statistics v23).

Statistical tests were chosen according to normal practices for scientific methods (Peacock and Kerry, 2006). Normal distribution was tested by the Shapiro-Wilks test. If the data were found to be normally distributed (parametric), correlation was performed by the Pearson correlation and comparison of groups was undertaken by a t-test. If the data were non-normally distributed

(non-parametric), Spearman correlation and Mann Whitney U test for comparison of groups were used. Significance was determined at $p<0.05$.

2.2.2.1 Regression analysis

Regression analysis was undertaken to describe the influence of independent variables (i.e. patient age and sex, prescribed dose or sample time post dose) on a dependent variable (e.g. oral fluid analyte concentration). A model was created with each relevant independent variable included, and the most non-significant independent variable removed sequentially until all remaining in the model were significant to $p<0.01$ (Peacock and Kerry, 2006).

The model created expressed the relative impact of each significant ($p < 0.01$) independent variable (V) on the dependent variable (Y, Table 2.1):

- R: correlation between the observed result, and that predicted by applying the regression model.
- R^2: degree of association between the observed result and that predicted by the model.
- B: relationship between a change in the independent variable and change in dependent variable – for an increase in V of 1, the dependent variable Y will increase by B. The 95 % confidence intervals (CI) of B describe the spread of results.
- Beta: magnitude of influence of V within the model predicting Y – this expresses the relevance of this independent variable to the dependant variable.

The regression model can be summarised according to the equation:

$$Y = (B_1 \times V_1) + (B_2 \times V_2) + B_c$$

Table 2.1: Summary of presentation of the results from a regression analysis model

Dependent variable	Model (R^a, $R^{2\,b}$)	Significant independent variables	Predictor			
			B [c]	95 % CI	Beta [d]	*P* value
Y	R R^2	V1	B_1	CI_1	$Beta_1$	p_1
		V2	B_2	CI_2	$Beta_2$	p_2
		constant	B_c	CI_c	-	p_c

a – correlation between observed and predicted result
b – proportion of variance in the dependent variable that can be explained by applying the model, degree of association between observes and predicted results
c – coefficient for predicting the dependent variable from the independent variable
d – standardised coefficient to describe the magnitude of effect of each independent variable

The strength of the model, described by R and R^2, explains the proportion of the dependent variability that can be predicted by the independent variables, and therefore describes the relevance of the model.

2.2.3 Dataset creation

Initial analyses were undertaken using the entire dataset. Analyte concentration (C) was corrected for dose giving plasma concentration per mg quetiapine prescribed daily (C/D).

Samples where no quetiapine was detected in the plasma were analysed separately, and removed from a regression data subset. In addition, results from patients from whom multiple samples were received were averaged to give a single result per patient to reduce the possible effect of skew from any patient from whom multiple samples were received (Fisher *et al*., 2012A). Using this subset, correlation and regression analysis was undertaken and metabolic ratio (MR) was calculated as plasma concentration of the metabolite divided by plasma quetiapine concentration.

To investigate the impact of co-prescribed medications, it was assumed that where one or more medications other than quetiapine were listed on the request form that this corresponded to all medications prescribed. Therefore, a subset of results was formed that included only those results from patients where at least one medication was listed. Using this subset of data, the impact of individual co-prescribed medications investigated, using the group of results that did not list that relevant medication as a control group.

2.3 Results

There were 509 samples from 321 patients (175 male, 146 female; Table 2.2) with an overall median (range) age of 37 (13-86) yr.

2.3.1 Summary of quetiapine concentrations

The summary concentrations attained for the samples as well as the dose and time since last dose are given in Table 2.3. The relationship between plasma analyte concentration and dose was investigated and showed whilst there was a trend of an increase in plasma metabolite concentrations with increasing dose, this was not matched for quetiapine (Figure 2-2). Correcting the analyte concentrations for quetiapine dose (Table 2.4) reduced the variation in the results observed by 0.5-2 fold when comparing the differences between the 5^{th} and 95^{th} percentiles (C/D compared to C).

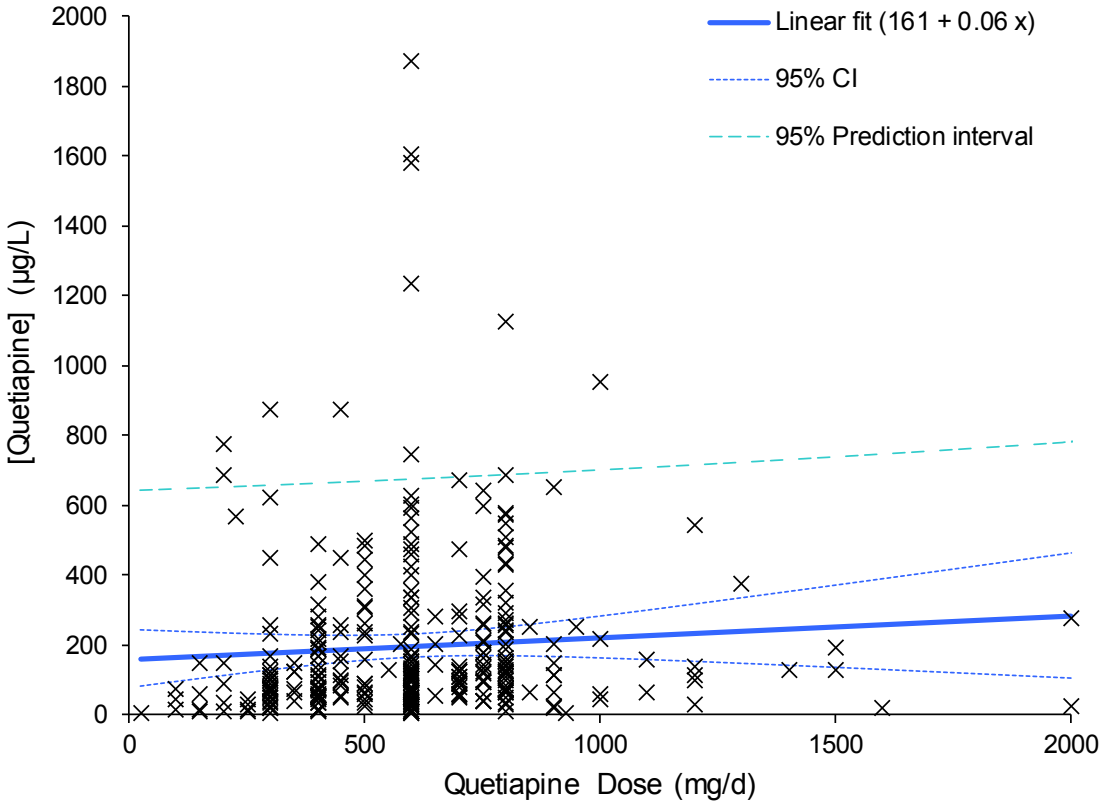
Table 2.2: Quetiapine audit: Patient demographics of quetiapine samples

	Age (years)	
	Male	Female
Minimum	15	13
5th Percentile	17	16
25th Percentile	27	28
Median	36	39
75th Percentile	46	51
95th Percentile	60	65
Maximum	73	86
Mean	37	40
SD	13	15
N	*175*	*146*

Table 2.3: Quetiapine audit: Summary analyte concentrations and dose

	Concentration (µg/L)				Dose (mg/d)	TSLD (h)
	Quetiapine	*N*-Desalkyl-quetiapine	*O*-Desalkyl-quetiapine	7-Hydroxy-quetiapine		
Minimum	5	5	2	2	25	8
5th Percentile	14	22	3	2	250	11
25th Percentile	59	77	8	4	400	12
Median	124	133	14	7	600	13
75th Percentile	280	225	25	14	750	15
95th Percentile	679	380	51	38	1000	20
Maximum	2980	621	157	118	2000	26
Mean	228	161	19	12	601	14
SD	300	113	19	14	262	3
Not detected	*33*	*26*	*41*	*94*		
N	509	509	509	509	335	287

Figure 2-2: Quetiapine audit: Plasma analyte concentration and dose relationship
Trendlines, 95 % confidence and prediction intervals shown.



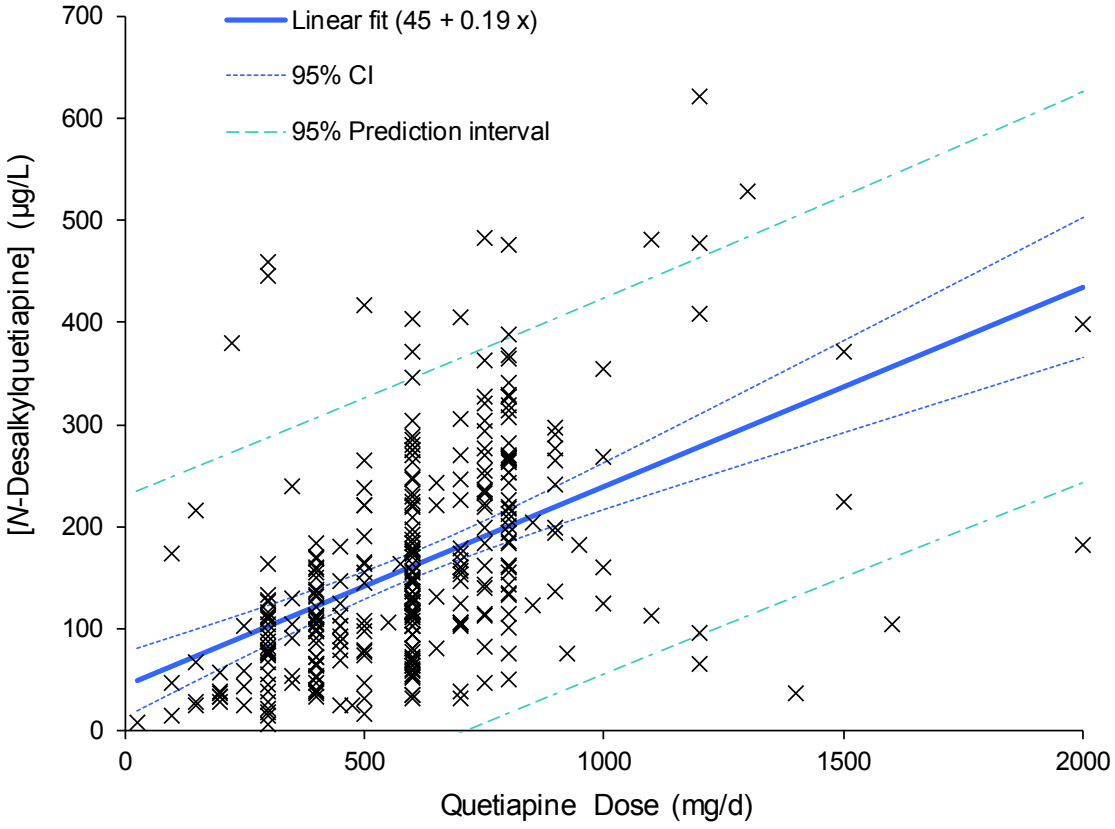
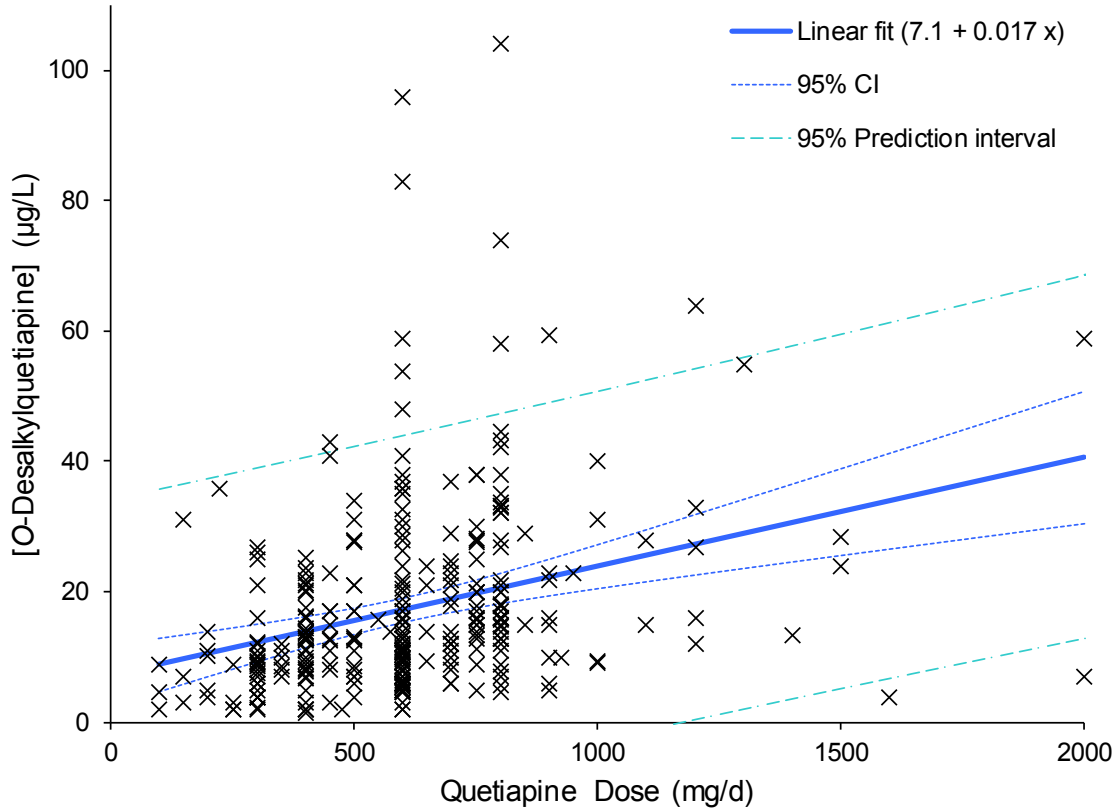


Figure 2-2 (cont.)



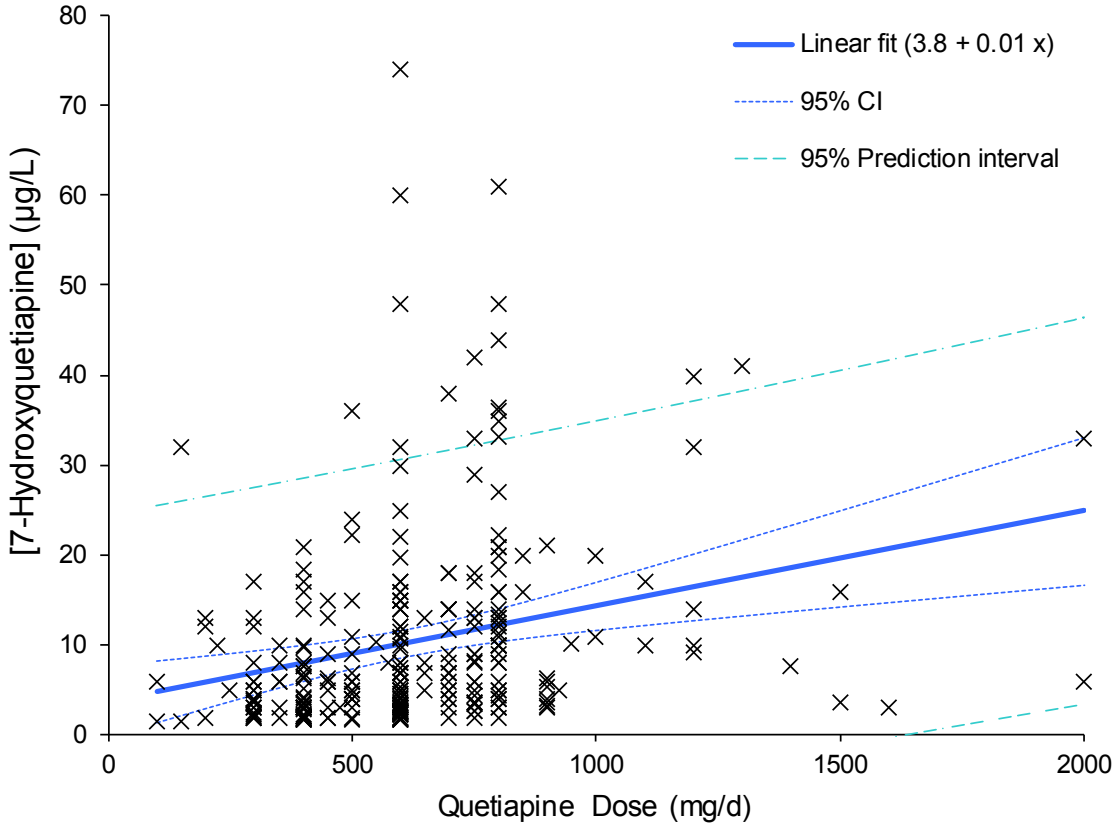


Table 2.4: Quetiapine audit: Summary dose-corrected analyte concentrations

	Dose-corrected concentration per mg quetiapine prescribed daily (µg/L / mg/d)			
	Quetiapine	*N*-Desalkyl-quetiapine	*O*-Desalkyl-quetiapine	7-Hydroxy-quetiapine
Minimum	0.01	0.03	0.003	0.002
5th Percentile	0.03	0.08	0.007	0.004
25th Percentile	0.11	0.17	0.016	0.006
Median	0.20	0.25	0.024	0.012
75th Percentile	0.45	0.35	0.038	0.020
95th Percentile	1.01	0.51	0.071	0.053
Maximum	3.89	1.74	0.207	0.213
Mean	0.37	0.29	0.031	0.018
SD	0.52	0.21	0.025	0.020
N	320	320	318	285

Investigating the concentrations that were attained at a given dose is important in interpreting an individual result with regards to expected concentrations (Table 2.5) as it establishes a likely range of concentrations enabling outlying concentrations to be highlighted and compliance to be assessed.

Plasma metabolite concentrations increased with increasing dose for the metabolites according to both the median and mean, however the plasma quetiapine concentrations were much more variable and did not show a steady increase with increasing dose.

As to the samples in which an analyte was not detected (ND, Table 2.5), these were from patients prescribed doses across the entire dose range, rather than just lower doses, suggesting that non-adherence to treatment may be the underlying cause of these findings in many cases rather than fast metabolism alone.

Table 2.5: Quetiapine audit: Plasma analyte concentrations by prescribed dose band.
ND = not detected

a) Quetiapine

Dose (mg/d)	N	ND	Quetiapine concentration (µg/L)								
			Min	5th	25th	Median	75th	95th	Max	Mean	SD
25-200	14	0	5	8	17	54	135	721	778	153	251
225-350	44	6	7	14	43	79	124	578	875	142	186
400-575	71	2	13	20	63	130	244	470	877	177	156
600	84	4	7	10	48	105	264	772	1875	234	359
650-750	48	0	40	41	93	128	236	557	671	185	154
800-950	56	2	6	27	76	152	288	606	1126	233	218
1000-2000	18	0	23	27	61	131	212	608	953	200	231

b) *N*-Desalkylquetiapine

Dose (mg/d)	N	ND	*N*-Desalkylquetiapine concentration (µg/L)								
			Min	5th	25th	Median	75th	95th	Max	Mean	SD
25-200	14	0	8	13	29	36	55	189	216	58	60
225-350	44	4	8	19	53	86	114	384	460	109	103
400-575	71	0	17	34	73	103	150	222	417	114	65
600	84	5	32	54	99	136	196	291	404	155	82
650-750	48	0	33	59	131	182	251	351	484	199	96
800-950	56	2	51	92	167	223	281	367	477	230	87
1000-2000	18	0	37	62	116	247	407	543	621	279	180

c) *O*-Desalkylquetiapine

Dose (mg/d)	N	ND	*O*-Desalkylquetiapine concentration (µg/L)								
			Min	5th	25th	Median	75th	95th	Max	Mean	SD
25-200	14	3	2	3	4	7	11	23	31	9	8
225-350	44	6	2	2	5	9	11	26	36	10	8
400-575	71	0	2	2	8	13	19	30	43	14	9
600	84	5	2	5	7	11	21	49	96	18	17
650-750	48	0	5	7	12	17	23	35	38	18	8
800-950	56	2	5	6	13	18	32	58	104	24	18
1000-2000	18	0	4	7	12	26	33	60	64	26	18

d) 7-Hydroxyquetiapine

Dose (mg/d)	N	ND	7-Hydroxyquetiapine concentration (µg/L)								
			Min	5th	25th	Median	75th	95th	Max	Mean	SD
25-200	14	7	2	2	2	6	13	26	32	10	11
225-350	44	16	2	2	3	4	7	13	17	6	4
400-575	71	5	2	2	3	5	9	20	36	7	6
600	84	13	2	2	3	6	13	31	74	10	13
650-750	48	2	2	3	5	8	14	32	42	11	9
800-950	56	5	2	3	5	11	20	40	61	15	13
1000-2000	18	2	3	3	9	13	23	40	41	17	13

2.3.1.1 Quetiapine not detected

Twenty-four samples were received that had plasma quetiapine <5 µg/L. Of these, *N*-desalkylquetiapine was present in 6 (range 5-26 µg/L), and only the sample with the highest *N*-desalkylquetiapine concentrations had *O*-desalkylquetiapine and 7-hydroxyquetiapine (2 and 3 µg/L, respectively) present. Prescribed quetiapine dose was given in 9 cases, with a median (range) of 475 (300-800) mg/d.

The results from all 24 samples were excluded from the final regression data subset.

2.3.2 Influence of studied variables on the concentrations

The final regression data subset contained 297 records (Table 2.6); dose was recorded in 180 instances (25 to 2000 mg/d), formulation for 49 results (10 IR, 39 ER), and time since last dose for 150 records (118 were samples taken 10 to 15 hours post-dose, with 32 over 15 hours post-dose).

The MR was calculated for each of the metabolites. The results are summarised in Table 2.7.

Median MR for *N*-desalkylquetiapine shows an overall one-to-one relationship to quetiapine, whereas the MR for *O*-desalkylquetiapine and 7-hydroxyquetiapine were 10-times and 20-times lower than quetiapine concentrations, respectively.

To establish whether there was a relationship between analyte concentrations and dose, age, or between the metabolite and quetiapine plasma concentrations, the correlations between these parameters were investigated (Table 2.8).

There was a significant relationship between the plasma concentration of each analyte and dose, although the correlations (0.23 to 0.56) were poor. There was also a significant correlation between the plasma metabolite concentrations (C and C/D) and plasma quetiapine concentration, the strongest correlation was to *O*-desalkylquetiapine and the weakest to *N*-desalkylquetiapine. In addition as age increased, dose significantly decreased, and there was a significant decrease in MR for 7-hydroxyquetiapine and *N*-desalkylquetiapine, and increase in quetiapine C/D.

The relationship between the metabolites showed the pairs *N*-desalkylquetiapine/ *O*-desalkylquetiapine and *O*-desalkylquetiapine/7-hydroxyquetiapine had the strongest relationship, especially when corrected for dose (Table 2.9).

Table 2.6: Quetiapine audit: Summary data subset analyte concentrations and dosage.

	Concentration (µg/L)				Dose (mg/d)	TSLD (h)
	Quetiapine	*N*-Desalkyl-quetiapine	*O*-Desalkyl-quetiapine	7-Hydroxy-quetiapine		
Minimum	5	5	2	2	25	8
5th Percentile	15	23	3	2	300	11
25th Percentile	66	77	9	5	400	12
Median	131	142	16	8	600	13
75th Percentile	295	234	26	14	800	15
95th Percentile	636	383	54	38	900	20
Maximum	2980	590	157	118	2000	26
Mean	235	164	20	13	611	14
SD	302	113	20	14	261	3
Not detected	0	0	6	37		
N (total)	297	297	291	260	180	151

Table 2.7: Quetiapine audit: Summary quetiapine metabolic ratio data

	Metabolic ratio (plasma concentration of metabolite / quetiapine)		
	N-Desalkylquetiapine	*O*-Desalkylquetiapine	7-Hydroxyquetiapine
Minimum	0.03	0.012	0.007
5th Percentile	0.18	0.047	0.015
25th Percentile	0.50	0.076	0.033
Median	0.99	0.103	0.050
75th Percentile	1.80	0.153	0.087
95th Percentile	4.89	0.264	0.200
Maximum	17.38	1.667	0.833
Mean	1.60	0.129	0.074
SD	2.21	0.125	0.084
N	*297*	*291*	*260*

Table 2.8: Quetiapine audit: Correlation between quetiapine concentration, dose and age for quetiapine and metabolites.
Significance (p values) of Spearman correlation given in brackets; significant correlations highlighted in bold.

Dependent variable *		Factor, correlation and significance		
		Quetiapine concentration, µg/L	Dose, mg/d	Age, years
Quetiapine	C	-	**0.23 (0.001)**	0.03 (0.638)
	C/D	-	-	**0.17 (0.021)**
N-Desalkylquetiapine	C	**0.47 (<0.001)**	**0.56 (<0.001)**	-0.09 (0.112)
	C/D	**0.32 (<0.001)**	-	-0.02 (0.748)
	MR	-	**0.15 (0.039)**	**-0.13 (0.020)**
O-Desalkylquetiapine	C	**0.86 (<0.001)**	**0.43 (<0.001)**	-0.01 (0.891)
	C/D	**0.77 (<0.001)**	-	0.12 (0.098)
	MR	-	**0.18 (0.013)**	-0.08 (0.169)
7-Hydroxyquetiapine	C	**0.66 (<0.001)**	**0.32 (<0.001)**	**-0.12 (0.044)**
	C/D	**0.54 (<0.001)**	-	-0.03 (0.706)
	MR	-	**0.23 (0.003)**	**-0.18 (0.004)**
Dose (mg/d)		-	-	**-0.19 (0.011)**

* Variables: C = analyte concentration (µg/L), C/D = analyte concentration corrected for dose (µg/L per mg/d), MR = analyte concentration divided by quetiapine concentration.

Table 2.9: Quetiapine audit: Relationship between metabolites
Significance (p values) of Spearman correlation given in brackets.

Metabolite pairs	Analyte correlation and significance *		
	C	C/D	MR
N-Desalkylquetiapine and *O*-Desalkylquetiapine	0.57 (<0.001)	0.64 (<0.001)	0.77 (<0.001)
N-Desalkylquetiapine and 7-Hydroxyquetiapine	0.49 (<0.001)	0.25 (0.001)	0.75 (<0.001)
O-Desalkylquetiapine and 7-Hydroxyquetiapine	0.71 (<0.001)	0.61 (<0.001)	0.80 (<0.001)

* C = analyte concentration (µg/L), C/D = analyte concentration corrected for dose (µg/L per mg/d), MR = analyte concentration divided by quetiapine concentration.

The effect of sex, formulation and time since last dose on the plasma concentrations was also investigated (Table 2.10). The plasma concentrations of quetiapine and *O*-desalkylquetiapine were significantly different between results from 10-15 hours post-dose and over 15 hours post-dose. There was a significant difference in *N*-desalkylquetiapine and *O*-desalkylquetiapine C/D between males and females, but there was not a significant difference in the prescribed dose between males and females. Formulation significantly affected quetiapine plasma concentration and C/D, *O*-desalkylquetiapine C/D, and the *N*-desalkylquetiapine MR.

To specifically investigate the effect of the different quetiapine formulations on quetiapine exposure, the concentrations, C/D and MR of quetiapine and its metabolites were tabulated (Table 2.11). As the group comparison showed (Table 2.10), there was no large difference in the dose prescribed between the two groups. Quetiapine C and C/D did differ, however, being approximately one-third in the group prescribed IR compared to ER, likely due to the short half-life of quetiapine influencing the results markedly in those prescribed the IR formulation.

Table 2.10: Quetiapine audit: Comparison for sex, formulation, and time since last dose for quetiapine and metabolites.
Significant differences according to Mann Whitney U tests are highlighted in bold.

Dependent variable *		Factor, significance between groups		
		Sex (M vs F)	Formulation (IR vs ER)	TSLD (10-15 h, > 15h)
Quetiapine	C	0.798	**0.029**	**0.045**
	C/D	0.130	**0.011**	0.106
N-Desalkylquetiapine	C	0.804	0.766	0.117
	C/D	**0.011**	0.402	0.355
	MR	0.687	**0.044**	0.076
O-Desalkylquetiapine	C	0.505	0.369	**0.021**
	C/D	**0.007**	**0.048**	0.154
	MR	0.219	0.052	0.181
7-Hydroxyquetiapine	C	0.241	0.245	0.122
	C/D	0.146	0.084	0.430
	MR	0.716	0.501	0.463
Dose (mg/d)		0.149	0.398	0.286

* Variables: C = analyte concentration (µg/L), C/D = analyte concentration corrected for dose (µg/L per mg/d), MR = analyte concentration divided by quetiapine concentration.

Table 2.11: Quetiapine audit: Effect of formulation on quetiapine metabolites
Presented as median, range (minimum to maximum) and the 5th, 25th, 75th and 95th percentiles, as well as the mean and standard deviation. * indicates one sample not detected. Groups indicated as different by Mann Whitney U test (Table 2.10) highlighted bold. N=10, IR; N=39, ER.

			Min	5th	25th	Median	75th	95th	Max	Mean	SD
	Quetiapine Dose	IR	25	300	413	575	725	900	900	555	254
		ER	300	300	381	550	600	900	800	521	156
	TSLD	IR	12	12	12	13	15	21	23	15	4
		ER	10	11	12	12	13	19	26	13	3
Concentration	Quetiapine	IR	5	27	63	***95***	114	365	419	134	126
		ER	15	33	105	***192***	368	1001	1729	311	343
	N-Desalkyl-quetiapine	IR	8	41	113	128	145	286	360	141	91
		ER	41	62	89	127	197	316	406	159	91
	O-Desalkyl-quetiapine*	IR *	9	10	13	14	19	26	29	16	6
		ER	3	9	13	18	24	54	58	22	14
	7-Hydroxy-quetiapine*	IR *	2	3	4	5	8	26	29	9	9
		ER	2	2	5	8	17	39	67	13	13
C/D	Quetiapine	IR	0.08	0.09	0.13	***0.18***	0.23	0.60	0.76	0.24	0.20
		ER	0.03	0.06	0.22	***0.46***	0.77	1.63	3.04	0.61	0.65
	N-Desalkyl-quetiapine	IR	0.12	0.15	0.22	0.26	0.29	0.41	0.48	0.26	0.10
		ER	0.07	0.10	0.18	0.30	0.39	0.49	0.58	0.30	0.13
	O-Desalkyl-quetiapine	IR	0.014	0.015	0.022	***0.028***	0.036	0.040	0.042	0.028	0.010
		ER	0.005	0.016	0.029	***0.035***	0.052	0.089	0.093	0.041	0.021
	7-Hydroxy-quetiapine	IR	0.004	0.004	0.006	0.009	0.014	0.042	0.052	0.015	0.015
		ER	0.003	0.007	0.010	0.017	0.029	0.058	0.112	0.024	0.022
MR	*N*-Desalkyl-quetiapine	IR	0.31	0.69	1.22	***1.37***	1.71	1.93	2.04	1.39	0.48
		ER	0.08	0.17	0.37	***0.73***	1.42	4.48	4.88	1.12	1.21
	O-Desalkyl-quetiapine	IR	0.054	0.071	0.115	0.133	0.170	0.213	0.218	0.142	0.052
		ER	0.018	0.039	0.065	0.095	0.132	0.232	0.382	0.109	0.069
	7-Hydroxy-quetiapine	IR	0.015	0.017	0.033	0.063	0.068	0.213	0.257	0.079	0.077
		ER	0.008	0.012	0.032	0.043	0.064	0.179	0.226	0.061	0.051

2.3.2.1 Regression analysis

To assess the influence of independent variables including age, sex, quetiapine dose, time since last dose and formulation, regression analyses were undertaken using the plasma quetiapine and metabolite concentrations as dependent variables (Table 2.12).

Plasma quetiapine concentration was not significantly influenced by any of the independent variables studied. Model strength was highest for *O*-desalkylquetiapine, where for an increase in plasma quetiapine concentration of 100 µg/L, *O*-desalkylquetiapine is predicted to increase by 4 µg/L. Quetiapine concentration was also a significant predictor of plasma 7-hydroxyquetiapine, predicting a 3 µg/L increase in 7-hydroxyquetiapine concentration for every 100 µg/L increase in quetiapine concentration, although the model strength was lower in this instance. Dose was the most significant predictor of plasma *N*-desalkylquetiapine concentration, where an increase in 100 mg/d quetiapine dose predicted an increase in *N*-desalkylquetiapine concentration of 17 µg/L. The other variables in each model predicted less than 30 % of the overall model strength.

Table 2.12: Quetiapine audit: Regression data for quetiapine metabolite concentrations

Dependent variable	Model * (R , R^2)	Significant independent variables	Predictor *			
			B	95 % CI	Beta	*P* value
Quetiapine concentration (µg/L)	Excluded sex (p=0.79), TSLD (p=0.75), age (p=0.86), dose (p=0.11), formulation (p=0.12)					
N-Desalkyl-quetiapine concentration (µg/L)	0.54, 0.29	Quetiapine dose (100 mg/d)	17.2	12.3, 22.0	0.45	<0.001
		Quetiapine concentration (µg/L)	0.12	0.06, 0.17	0.25	<0.001
		(constant)	36.1	3.0, 69.2	-	0.03
	Excluded TSLD (p=0.81), formulation (p=0.76), age (p=0.54), sex (p=0.52)					
O-Desalkyl-quetiapine concentration (µg/L)	0.81, 0.65	Quetiapine concentration (µg/L)	0.04	0.036, 0.046	0.73	<0.001
		Quetiapine dose (100 mg/d)	1.38	0.96, 1.81	0.29	<0.001
		(constant)	1.05	-1.9, 4.0	-	0.48
	Excluded formulation (p=0.91), age (p=0.55), TSLD (p=0.20), sex (p=0.19)					
7-Hydroxy-quetiapine concentration (µg/L)	0.70, 0.49	Quetiapine concentration (µg/L)	0.03	0.028, 0.036	0.69	<0.001
		(constant)	7.8	3.9, 11.7	-	<0.001
	Excluded dose (p=0.62), sex (p=0.65), formulation (p=0.28), TSLD (p=0.20)					

* model and predictors explained in section 2.2.2.1

Regression analysis was also undertaken for the analyte C/D and MR (Table 2.13). Quetiapine C/D was not significantly influenced by any of the independent variables studied, and *N*-desalkylquetiapine C/D had a very low model strength. Both *O*-desalkylquetiapine and 7-hydroxyquetiapine C/D were significantly predicted by quetiapine plasma concentration; an increase in quetiapine concentration of 700 µg/L predicted *O*-desalkylquetiapine C/D to increase by 0.1, and an increase in quetiapine concentration of 500 µg/L predicted 7-hydroxyquetiapine C/D to increase by 0.05. The MR could not be modelled for any metabolite.

Table 2.13: Quetiapine audit: Regression data for dose-corrected quetiapine metabolite concentrations and metabolic ratios

Dependent variable	Model * (R , R^2)	Significant independent variable	Predictor *			
			B	95 % CI	Beta	*P* value
Quetiapine C/D (µg/L per mg/d)	Excluded sex (p=0.44), formulation (p=0.32), TSLD (p=0.94), age (p=0.35)					
N-Desalkyl-quetiapine C/D (µg/L per mg/d)	0.27, 0.07	Quetiapine concentration (µg/L)	0.000	0.000, 0.000	0.24	0.001
		(constant)	0.308	0.238, 0.379	-	<0.001
	Excluded formulation (p=0.55), sex (p=0.56), age (p=0.97), TSLD (p=0.24)					
O-Desalkyl-quetiapine C/D (µg/L per mg/d)	0.77, 0.59	Quetiapine concentration (µg/L)	0.000	0.000, 0.000	0.73	<0.001
		Quetiapine dose (100 mg/d)	-0.002	-0.003, -0.001	-0.26	<0.001
		(constant)	0.029	0.023, 0.036	-	<0.001
	Excluded age (p=0.86), formulation (p=0.83), sex (p=0.33)					
7-Hydroxy-quetiapine C/D (µg/L per mg/d)	0.74, 0.55	Quetiapine concentration (µg/L)	0.000	0.000, 0.000	0.70	<0.001
		Quetiapine dose (100 mg/d)	-0.001	-0.002, 0.000	-0.15	0.007
		(constant)	0.017	0.010, 0.024	-	<0.001
	Excluded formulation (p=0.87), TSLD (p=0.60)					
N-Desalkyl-quetiapine MR	Excluded sex (p=0.92), age (p=0.46), formulation (p=0.32), TSLD (p=0.04), dose (p=0.03)					
O-Desalkyl-quetiapine MR	Excluded age (p=0.94), sex (p=0.58), TSLD (p=0.49), dose (p=0.58), formulation (p=0.16)					
7-Hydroxy-quetiapine MR	Excluded formulation (p=0.82), sex (p=0.74), age (p=0.55), TSLD (p=0.06), dose (p=0.03)					

* model and predictors explained in section 2.2.2.1

2.3.3 Co-prescribed medications

Based on the possible activity on relevant enzymes, or where an impact on plasma quetiapine concentrations has been reported, the list of prescribed medications was searched for: carbamazepine, phenytoin, levomepromazine, valproate, clozapine, fluvoxamine, fluoxetine, citalopram, lamotrigine and oxazepam.

There were no instances where phenytoin, levomepromazine, fluvoxamine, or oxazepam were mentioned.

There were 70 patients that listed at least one medication other than quetiapine; therefore, the results from these 70 patients were used for investigation of the impact of co-prescribed medications. Comparison of groups prescribed and not prescribed relevant medications showed that overall there was very little impact of the co-prescribed medications (Table 2.14), possibly due to the small sample numbers available to study.

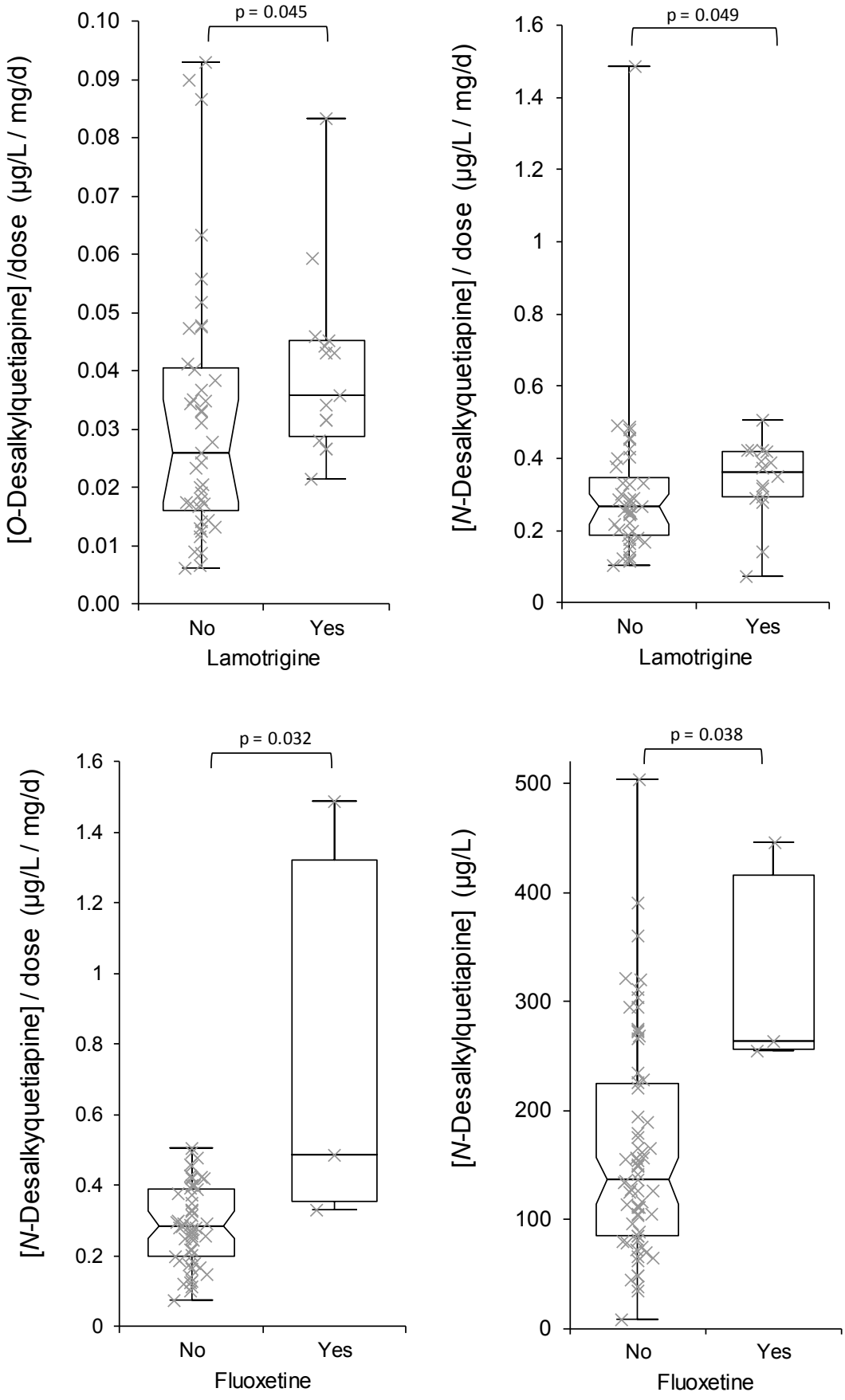
Those results from patients prescribed lamotrigine had significantly higher *N*-desalkylquetiapine and *O*-desalkylquetiapine C/D than those not prescribed lamotrigine, and the results from patients prescribed fluoxetine had significantly higher *N*-desalkylquetiapine C/D and plasma *N*-desalkylquetiapine concentration than those patients not prescribed fluoxetine (Figure 2-3).

Table 2.14: Quetiapine audit: Impact of co-prescribed medications
Significant differences according to Mann Whitney U tests are listed.

Co-prescribed medication	N	Significance between prescribed and non-prescribed groups *
Lamotrigine	19	*O*-Desalkylquetiapine C/D (p = 0.045) *N*-Desalkylquetiapine C/D (p = 0.049)
Valproate	15	None
Citalopram	6	None
Fluoxetine	3	*N*-Desalkylquetiapine C/D (p = 0.032) *N*-Desalkylquetiapine C (p = 0.038)
Carbamazepine	3	None
Clozapine	2	None

* C = analyte concentration (µg/L), C/D = analyte concentration corrected for dose (µg/L per mg/d).

Figure 2-3: Quetiapine audit: Significant co-prescribed medication interactions
Box = median and inter-quartile range, whiskers = range; Mann Whitney U p-value given between results prescribed the relevant medication compared to the rest of the subset as per Table 2.14.



2.4 Discussion

2.4.1 Plasma analyte concentrations and dose

The strongest correlation found was between plasma quetiapine and *O*-desalkylquetiapine, which is in agreement with previous work (Fisher *et al*., 2012A), and between *O*-desalkylquetiapine and 7-hydroxyquetipiapine. This may suggest a close metabolic pathway between these analytes, or be related to the fact these metabolites are believed to be metabolised by minor enzymes rather than the major route through CYP 3A4.

The table listing the plasma concentrations of quetiapine and its metabolites (Table 2.5) shows that for an increasing prescribed dose the median concentrations increased in line with dose, but there was much variation. These data will be helpful in assessing adherence, for example, in future quetiapine TDM samples.

Correcting the plasma analyte concentrations for dose reduced the sample to sample variability (taken as the difference between the 5^{th} and 95^{th} percentiles) for quetiapine down to a 30-fold variability, similar to the results found in other work (Bakken *et al*., 2011; Hasselstrøm and Linnet, 2004). As with this other work, the variability in quetiapine C/D was higher than that for *N*-desalkylquetiapine C/D, a finding thought to be related to the shorter half-life of quetiapine (Bakken *et al*., 2011), which would fit with our further findings that the variability for *O*-desalkylquetiapine and 7-hydroxyquetiapine C/D was between that for quetiapine and *N*-desalkylquetiapine C/D (8-fold, 12-fold, 19-fold, and 6-fold variability at 5^{th} and 95^{th} percentiles, respectively).

Regression analysis showed that the variability in *O*-desalkylquetiapine and 7-hydroxyquetiapine plasma concentrations could be predicted most strongly by plasma quetiapine concentration. An increase in quetiapine dose predicted an increase in *N*-desalkylquetiapine plasma concentration, although the model strength was lower in these instances. The relationship between the plasma analyte concentrations and dose was significant, but poor, with the best relationship between quetiapine and *N*-desalkylquetiapine (0.56). These results compare well with those calculated in the previous audit (Fisher *et al*., 2012A), and agree with the findings of Bakken *et al*. (2011).

The variability in quetiapine and *N*-desalkylquetiapine C/D was not significantly influenced by any of the independent variables, although *O*-desalkylquetiapine and 7-hydroxyquetiapine C/D were predicted to increase with an increase in plasma quetiapine concentration.

2.4.2 Metabolic ratio

Median MR showed overall that *N*-desalkylquetiapine reached equivalent plasma concentrations to quetiapine, while *O*-desalkylquetiapine was ten-times lower and 7-hydroxyquetiapine twenty-times lower than plasma quetiapine concentrations. MR could not be modelled for any metabolite, although a correlation was found where MR increased with increasing dose, matching the finding of Bakken *et al*. (2011).

2.4.3 Age and sex

Increasing age correlated to a decrease in prescribed quetiapine dose, MR of *N*-desalkylquetiapine and 7-hydroxyquetiapine, and in 7-hydroxyquetiapine concentration, but an increase in quetiapine C/D. This agrees with the results from other studies where quetiapine C/D was higher in patients aged over 65 or 70 (Bakken *et al*., 2011; Castberg *et al*., 2007; Hasselstrøm and Linnet, 2004; Wittmann *et al*., 2010). Higher plasma quetiapine concentrations were attained at the same dose as age increased, suggesting a reduced metabolic capacity possibly related to reduced hepatic blood flow; this suggestion is supported by a decreasing MR for both *N*-desalkylquetiapine and 7-hydroxyquetiapine with increasing age.

Neither age or sex were significant predictors of the variation in plasma analyte concentrations, analyte C/D or analyte MR.

There was no difference between males and females for prescribed quetiapine dose, plasma quetiapine concentration or quetiapine C/D, however there was a significant difference in C/D for both *N*-desalkylquetiapine and *O*-desalkylquetiapine. One group suggested quetiapine C/D was higher in females, though not when weight corrected (Hasselstrøm and Linnet, 2004). However, the results presented here agree with other work where no differences were found between males and females (Bakken *et al*., 2011; Handley *et al*., 2013)

2.4.4 Formulation

Formulation was recorded for 49 patients. Quetiapine plasma concentration, quetiapine C/D and *O*-desalkylquetiapine C/D were significantly higher in patients prescribed ER formulation that IR formulation, and *N*-desalkylquetiapine MR was significantly lower (Table 2.11). The rate of

liberation of quetiapine from the ER formulation is the reason why the quetiapine plasma concentration and C/D is affected by formulation. *O*-Desalkylquetiapine and 7-hydroxyquetiapine plasma concentrations and C/D were also higher in the ER formulation group, although this difference was not always significant. *N*-Desalkylquetiapine was much less affected by formulation, with very little difference between both plasma concentration and C/D. Therefore, it followed that the MR was affected due to the impact on quetiapine, likely due to the short plasma half-life of quetiapine, not being matched by a similar impact on *N*-desalkylquetiapine with its longer plasma half-life (7 and 11 hours, respectively).

2.4.5 Time since last dose

Time since last dose was recorded in 150 cases, with 118 samples taken between 10 to 15 hours, and 32 samples taken over 15 hours post-dose. Between the two groups (10 to 15 hours post-dose and over 15 hours post-dose) there was only significant difference in the quetiapine and *O*-desalkylquetiapine plasma concentrations, which is in line with the short plasma half-life of quetiapine and close relationship between quetiapine and *O*-desalkylquetiapine. These results match those where quetiapine C/D in samples taken 10 hours post-dose were higher than those taken 14 hours post-dose, and they found no difference in *N*-desalkylquetiapine C/D (Bakken *et al*., 2011).

2.4.6 Co-prescribed medications

Metabolism of quetiapine occurs primarily by CYP 3A4, with CYP 3A5 and CYP 2D6 playing a minor role (Figure 2-1). It would therefore be expected that polymorphisms of these enzymes and relevant transporters such as PGP could affect quetiapine exposure, hence the presence of co-prescribed medications which impact the activity of relevant enzymes are also likely to impact quetiapine exposure.

Agreement on the impact of polymorphisms on quetiapine exposure is not conclusive; polymorphisms in CYP 3A4 have been reported to affect quetiapine exposure (van der Weide and van der Weide, 2014), affect *N*-desalkylquetiapine but not quetiapine exposure (Bakken *et al.*, 2015), or have no impact on either quetiapine or *N*-desalkylquetiapine exposure (Nikisch *et al.*, 2010), although sample numbers were small in this latter study. CYP 2D6 polymorphisms were said to have no impact on either quetiapine or *N*-desalkylquetiapine concentrations in a small study (Nikisch *et al.*, 2010), and an impact only on *N*-desalkylquetiapine exposure in a

larger study (Bakken *et al.*, 2015). Polymorphisms of CYP 3A5 may affect quetiapine pharmacokinetics (Kim *et al.*, 2014), possibly through the formation of other minor metabolites, such as *O*-desalkylquetiapine (Bakken *et al.*, 2009), although an effect was not seen on either quetiapine or *N*-desalkylquetiapine exposure in another study (Bakken *et al.*, 2015). Quetiapine is also believed to be transported across cell membranes and also across the blood-brain barrier by PGP, but the presence of a polymorphism in the encoding gene (ABCB1) was not found to have significant effects on plasma quetiapine concentrations in some studies (Bakken *et al.*, 2015; Kim *et al.*, 2014), although another report suggests ABCB1 genetic polymorphisms play a large part in quetiapine exposure (Nikisch *et al.*, 2010).

With regards to non-genetic effects on CYP activity, the results are again inconclusive, possibly due to small sample numbers in the studies. Lower quetiapine concentrations have been observed in patients co-prescribed CYP 3A4 inducers such as carbamazepine and phenytoin, either as a reduction in quetiapine C/D (Bakken *et al*., 2011; Castberg *et al.*, 2007; Hasselstrøm and Linnet, 2004) or an increase in quetiapine clearance (Isbister *et al.*, 2007; Wittmann *et al.*, 2010; Wong *et al*, 2001). *N*-Desalkylquetiapine C/D was also found to be slightly lower in patients prescribed CYP 3A4 inducers (Bakken *et al*., 2011). Co-ingestion of CYP 3A4 inhibitors decreased quetiapine clearance (Isbister *et al.*, 2007), and co-prescription of valproate, recorded as an inhibitor of both CYP 3A4 and CYP 2D6, significantly increased quetiapine concentrations (Aicchorn *et al.*, 2006). Drugs that interact with CYP 2D6, including levomepromazine, did not display higher quetiapine C/D than the remainder of the group (Hasselstrøm and Linnet, 2004). Other medications investigated showed that co-prescription of clozapine, fluvoxamine and citalopram were associated with increased quetiapine C/D, whereas lamotrigine and oxazepam decreased quetiapine C/D (Castberg *et al.*, 2007).

In this work, there were only a limited number of cases which referred to co-prescribed medications, and therefore this limited statistical significance. Only 2 medications impacted any of the results: patients prescribed lamotrigine had significantly higher *N*-desalkylquetiapine and *O*-desalkylquetiapine C/D than the group not prescribed lamotrigine, and the patients prescribed fluoxetine had significantly higher *N*-desalkylquetiapine C/D and plasma *N*-desalkylquetiapine concentration. Multiple concurrent medications and possible dose-related influence, neither of which could be studied, add to the complexity of predicting the influence of co-prescribed medication in an individual.

An impact of lamotrigine in decreasing quetiapine C/D has been reported, although a mechanism for such an effect was not discussed (Castberg *et al*., 2007). It is possible that lamotrigine interaction was through PGP inhibition (Weiss *et al*., 2003) or tissue-specific CYP 3A4 inhibition (Perucca, 2005). Whatever the route, it seems that clearance of *N*-desalkylquetiapine and *O*-desalkylquetiapine is inhibited by lamotrigine without having a significant impact on either quetiapine or 7-hydroxyquetiapine clearance, possibly related to this metabolite being metabolised primarily by CYP 2D6.

In the present study, fluoxetine was found to increase the *N*-desalkylquetiapine C and C/D, but had no impact on the plasma quetiapine concentrations or other metabolites studied. Fluoxetine is a CYP 2D6 inhibitor, although norfluoxetine may be a 3A4 inhibitor (Hemeryck and Belpaire, 2002). It is believed that *N*-desalkylquetiapine is formed through CYP 3A4 activity (Figure 2-1), therefore norfluoxetine may have a larger impact than previously shown, or inhibition of CYP 2D6 may inhibit the minor metabolic route leading to an increased proportion of the dose giving rise to *N*-desalkylquetiapine.

2.4.7 Application of the metabolites in quetiapine TDM

Previous work has shown that there is no clear evidence of a target range for plasma quetiapine with respect to clinical response (Sparshatt *et al*., 2011).

This work has shown that analysis of the quetiapine metabolites can provide further information in plasma quetiapine TDM. Measurement of *N*-desalkylquetiapine has been shown to be helpful in assessing quetiapine exposure, at least in part due to the reduced variability between samples due to its longer plasma half-life. The other two metabolites investigated here have a similar though slightly longer plasma half-life to quetiapine, and *O*-desalkylquetiapine shows a very close relationship to quetiapine. Age plays a significant role in either the formation, or clearance of 7-hydroxyquetiapine, possibly more so than quetiapine or the other metabolites. Plasma concentrations of the quetiapine metabolites cannot be predicted by age, sex, dose or time since last dose.

This work has established dose-related plasma concentration ranges for quetiapine and the metabolites studied, and highlighted the different expected concentrations attained in samples from patients prescribed IR and ER quetiapine. The variability in plasma quetiapine concentrations as established by the dose-related concentration data supports the use of

metabolites to assess quetiapine exposure, since the metabolite plasma concentrations were found to increase with increasing dose, a finding not matched by study of quetiapine itself.

It should be noted that one limitation of the audit undertaken is that these samples are routine TDM samples, therefore the reason that these analyses were requested is not known. There may be cases where the patient has become stabilised on a dose at which they are responding to, and TDM is undertaken to establish the steady state stable concentrations for future comparisons; However, there may also be cases where the patient is not responding to the medication and adherence is being assessed. As such, the concentrations reported may not correlate to a reference range for clinical response.

One possible way to achieve a greater benefit from application of the quetiapine metabolites is to undertake an audit of only those results obtained from patients known to be responding clinically to the medication. This investigation could be extended to subdivide response to the different diagnoses such as successful treatment of schizophrenia, depression, anxiety and sleep disorder. To obtain a dataset of samples only from those patients who are responding to the treatment would require a clinical trial to be undertaken across a large number of units; however the benefits to this effort could enable therapeutic reference ranges of quetiapine and its plasma metabolites to be established in order to guide quetiapine therapy in the future and improve treatment success in these patients.

The aims of this work have been achieved, a dataset was created and the results analysed. Whilst studied alone the metabolites may not provide conclusive evidence of quetiapine exposure, but they can provide further information to help interpret plasma quetiapine TDM results. Overall, the evidence suggests an ongoing benefit to quantifying these additional metabolites, and further work looking at the concentrations in line with clinical response, both for schizophrenia and in depressive disorders, could enable reference ranges for quetiapine and the metabolites studied to be established. Dose-related plasma quetiapine metabolite concentrations have been presented herein and can be used to interpret future plasma quetiapine metabolite results.

Chapter 3. Potential role of oral fluid in antipsychotic TDM: method development and assessment

3.1 Introduction

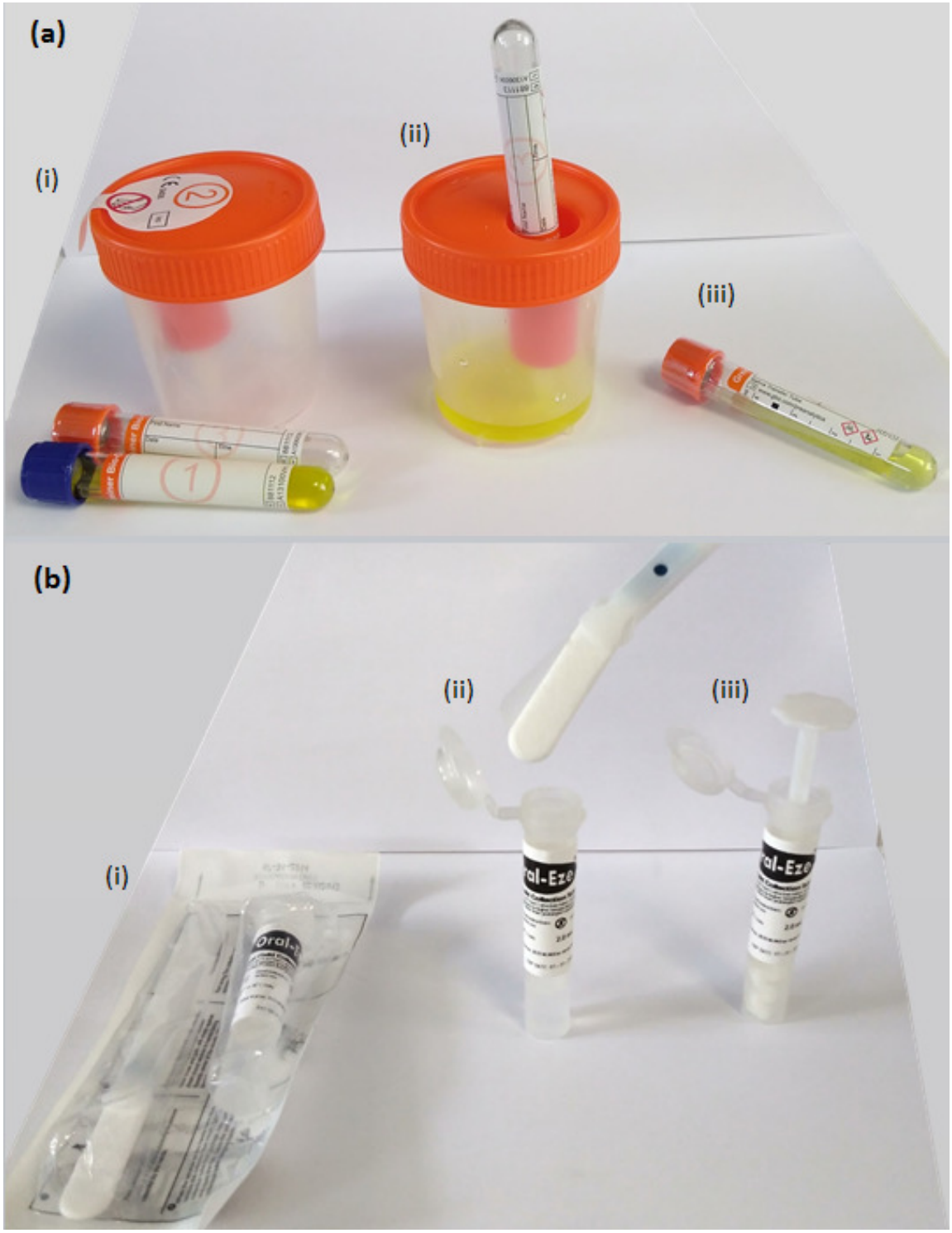
The technique of oral fluid collection can vary from the drool method (unstimulated pooling of oral fluid in the mouth and spitting into a tube), collection into a buffered liquid held in the mouth, or collection using an adsorbent pad held in the mouth that is either untreated, or treated for example with an acidic buffer to further stimulate oral fluid flow. With each collection technique, there are different analytical considerations, including the pH of the fluid collected, analyte recovery from a pad, and compensation for any dilution effect during sample collection.

The two collection devices used in this study were the GBO and Oral-Eze collection systems (Figure 3-1). Both devices use a stimulated collection, for the GBO device a buffered solution is held in the mouth and subsequently collected for analysis and for the Oral-Eze device an adsorbant pad is held in the mouth and then stored post-collection within a buffered solution. These differing collection techniques will allow comparison of the in-mouth buffering device against one that collects only moderately stimulated oral fluid in uncontrolled oral cavity pH. Therefore the theoretical improvement proposed from the buffering to reduce between patient and between collection oral fluid pH can be directly assessed.

3.1.1 GBO oral fluid collection device

The GBO device consists of four components: (1) a mouth wash that is used immediately prior to OF collection, (2) 4 mL of collection buffer containing a food dye (tartrazine) that is held in the mouth for 2 minutes, (3) a beaker into which the buffer solution is spat, and (4) two tubes containing the preservative sodium azide that are used to securely collect the sample from the beaker to be sent for analysis (Greiner Bio One, 2011; Figure 3-1). Tartrazine contained within the collection buffer is used to quantify the amount of oral fluid contained within the final collection fluid by calculation of the dilution of the original tartrazine concentration, and sodium citrate is used to buffer the solution during collection (approx. pH 4-6) and to stimulate oral fluid flow.

Figure 3-1: Oral fluid collection devices images.
(a) GBO collection device: i) collection buffer, collection beaker and saliva transfer tubes, ii) once the collection buffer has been held in the mouth for 2 minutes, the solution is spat into the beaker, and the saliva transfer tube is inserted into the lid to take up the sample securely, iii) the sealed saliva transfer tube can then be stored or analysed directly.
(b) Oral-Eze collection device: i) the collection device is attached to a handle and received with a collection buffer tube, ii) the pad is held against the cheek until the indicator window turns blue or at 10 minutes and then the pad is dispensed off the handle into the collection buffer, iii) the pad is plunged down to enable the collection fluid to be analysed directly or stored.



Accurate measurement of tartrazine within the collected fluid is a vital part of accurate analyte quantification, whether within a single method together with the drugs under study or separately, since the degree of dilution of the oral fluid collection solution varies from sample to sample.

3.1.2 Oral-Eze oral fluid collection device

The Oral-Eze device consists of an adsorbant collection pad of untreated pure cotton fibre that is held in the mouth until the indicator on the handle turns blue to indicate that 1 mL oral fluid has been collected (or for a maximum of 10 minutes). The pad is then detached into a tube which contains 2 mL of a buffer (approx. pH 4-6) for storage until analysis (Thermo Fisher Scientific, 2013; Figure 3-1).

Quantification of the analytes from this sample must take into account the 1+2 dilution of oral fluid into the buffer post-collection. This device works on the assumption that a fixed volume of oral fluid is collected, and therefore no further compensation for sample volume is required, although possible loss of analyte via adsorption onto the pad must be taken into account.

3.1.3 Analytical considerations

Oral fluid samples collected using both collection devices are stored under acidic conditions in a buffer at approximately pH 4-6; hence this must be taken into account during sample preparation if pH dependent extraction is undertaken.

Assessment of whether a method is fit-for-purpose is a critical part of implementing a new method, as it is necessary to show that a method is accurate and precise, as well as sensitive enough to detect the analytes in question and selective enough that interferences are known or minimised where possible; in essence ensuring that the results reflect the concentration of the analyte in the relevant matrix at the time of sample collection, and that results are reproducible and reliable. For liquid chromatographic-mass spectrometric (LC-MS) assays it is important to ensure that the effects of ion suppression/enhancement sample to sample are assessed and minimised (Matuszewski *et al*., 2003).

The value of quetiapine metabolite assay in quetiapine has been discussed in Chapter 2, therefore these were included in the analytes studied.

3.1.4 Aims of the chapter

The established methodology for the measurement of atypical antipsychotics in plasma (Fisher *et al*., 2013B; Fisher *et al*., 2012B) will be combined and extended to include clozapine and norclozapine, and fluoxetine and norfluoxetine, to detect all analytes within a single analytical procedure.

Methods for the analysis of tartrazine in the GBO collected fluid will be investigated, first in a combined method with the analytes of interest so that only a single step is required for the patient sample analysis, and if this is unsuccessful methods for a separate method will be investigated.

The sample preparation procedure will be modified in order to facilitate use with oral fluid, taking into account the larger sample volume available as compared to plasma and the acidic nature of the buffered collection fluid. Separate methods will be created if necessary for the extraction from each oral fluid collection device to account for the different collection processes.

Both oral fluid methods will be assessed to ensure they are fit-for purpose.

3.2 Materials and instrumentation

3.2.1 Materials

Aripiprazole and dehydroaripiprazole were from Bristol-Myers Squibb (New York, USA). *Bis*-quetiapine fumarate was from AstraZeneca (London, UK). Clozapine and norclozapine were from Novartis (Frimley, UK). Olanzapine was from Eli Lilly (Indianapolis, USA). Risperidone and (±)-9-hydroxyrisperidone were from Janssen (Beerse, Belgium). Quetiapine-D_8 fumarate, (±)-amisulpride-D_5, dehydroaripiprazole-D_8, fluoxetine, norfluoxetine, fluoxetine-D_6, quetiapine, *N*-desalkylquetiapine, *N*-desalkylquetiapine-D_8, *O*-desalkylquetiapine, 7-hydroxyquetpiapine, 7-hydroxyquetpiapine-D_8, norfluoxetine-D_5, risperidone-D_4, (±)-9-hydroxy-risperidone-D_4 and (±)-sulpiride-D_3 and were from LGC Standards (Teddington, UK). Aripiprazole-D_8 was from Medical Isotopes (Pelham, USA). Clozapine-D_8, norclozapine-D_8, and olanzapine-D_3 were from Toronto Research Chemicals (Ontario, Canada). Butyl acetate, butanol and methanol (all HPLC grade) were from Rathburn (Walkerburn, Scotland). Ammonium acetate, tris(hydroxymethyl)-aminomethane (Tris; Trizma, >99.5 %), (±)-amisulpride, (±)-sulpiride, tartrazine, and newborn calf serum were from Sigma-Aldrich (Poole, UK). Acetic acid (Fluka ACS reagent), ammonia (28 %) and hydrochloric acid (both BDH AnalaR grade) and low-volume (max 1 mL) cuvettes

were from VWR (Lutterworth, UK). Pooled human dipotassium EDTA plasma was from Sera Laboratories International (West Sussex, UK). GBO collection systems, quantification kits and the blank buffer solution were donated by Greiner Bio-One (Stonehouse, UK). Oral-Eze collection system kits and blank buffer solution were donated by Thermo (Loughborough, UK). Blank oral fluid was collected according to the protocol for each collection system from drug-free volunteers and stored at -18 to-20 ºC until required. Polypropylene 2 mL tubes were from Alphalabs (Eastleigh, UK). Eppendorf flip-cap centrifuge tubes 1.5 mL were from Elkay (Basingstoke, UK). Autosampler vials 0.5 mL were from Sarstedt (Leicester, UK). Dreyer tubes, 60.5 x 7.5 mm i.d. glass test tube, were from Esslab (Essex, UK). 0.45 µm nylon Phenex filters were from Phenomenex (Macclesfield, UK). The pH probe was a LIQ-GLASS BNC combination electrode (Hamilton; Bonaduz, Switzerland) calibrated against aqueous buffers (pH 4, 7 and 10, Merck, Darmstadt, Germany). Water was deionised (Purite Select) to resistivity greater than 12 MΩ/cm.

The LC eluent was ammonium acetate (50 mmol/L) in methanol, filtered, and adjusted to apparent pH 6.0 with acetic acid.

The pH of the oral fluid collection device solutions and samples were tested using pH strips (Fisherbrand pH indicator paper sticks range 0-14, Fisher Scientific).

3.2.2 Instrumentation

The LC pump (PU-1580), column oven (CO-2067) and autosampler (AS-950) were from Jasco (Great Dunmow, UK). The guard cartridge and analytical column (stainless steel, 10 and 100 x 2.1 mm i.d., respectively) were packed with Waters Spherisorb S5SCX 5 µm sulfopropyl-modified silica (Hichrom, Reading, UK). The eluent flow-rate was 0.5 mL/min. Analytes were measured using selected reaction monitoring (SRM; sum of two product ions) following positive mode atmospheric pressure chemical ionisation (APCI; TSQ Quantum Access, ThermoFisher Scientific, Hemel Hempstead, UK). Quantification was by the sum of the peak area of both transitions as a ratio to the sum of the peak area of both transitions of the corresponding internal standard. Other ionisation source settings were: corona discharge current 4 µA; vaporiser and capillary temperatures 320 and 300 °C, respectively; auxiliary, sheath and ion sweep gas settings 5, 40, and 0 arbitrary units, respectively (Fisher *et al*., 2013B). High purity argon was used as collision gas (1.5 mTorr). Data acquisition and processing were via Xcalibur (version 2.0.7, ThermoFisher Scientific).

The spectrophotometer used for tartrazine assay was from Jenway (model 6315; Stone, UK) and data were recorded directly from the on-screen readout of the extinction at 450 and 520 nm.

3.3 Method development for tartrazine quantification in the GBO system

Successful use of the GBO system relies on the accurate quantification of tartrazine within the collection fluid. Tartrazine is a polar, hydrophilic dye, therefore analysis was not possible using LLE with SCX chromatography. Thus, different chromatographic techniques were investigated.

3.3.1 Analysis of tartrazine by MS detection

Reversed phase (RP) chromatography was investigated using a methanol-water gradient and an ACE C18 column (100 x 2.1 mm, 3 µm packing, flow rate 0.5 mL/min).

Sunset yellow was investigated simultaneously as an IS for tartrazine quantification since it is a similar compound in chromophore and size (molar mass 452.37 g/mol). Mass spectrometric conditions established for the quantification of tartrazine and sunset yellow were investigated. The tartrazine precursor ion would be expected to be based on the molar mass (534.36 g/mol) however since tartrazine has sodium present the precursor ion was found to be different in two papers both using electro-spray ionisation (ESI) with detection by either MS or MS/MS (Ma *et al*., 2006; Ates *et al*., 2011). Ma *et al*., 2006, used single quadrupole MS in negative ionisation mode at mass ions for tartrazine of 467 $[M\text{-}3Na+2H]^-$ and 423 $[M\text{-}3Na+2H\text{-}CO_2]^-$, and for sunset yellow 407 $[M\text{-}2Na+H]^-$ and 429 $[M\text{-}Na]^-$. Ates *et al*., 2011, used an MS/MS method using precursor ions 470.87 in positive ionisation mode monitoring no products for tartrazine, and 407.01 to product ions 206.55 and 142.35 for sunset yellow in negative ionisation mode.

Given the disparity in these papers between positive and negative ionisations for tartrazine, both of these ionisation polarities were investigated. Infusing directly into the instrument using ESI and APCI, in both positive and negative polarities gave no distinct predominant *m/z* peaks for either analyte at the ranges investigated, suggesting this analysis was not viable under the conditions studied.

3.3.2 Analysis of tartrazine by LC with UV detection

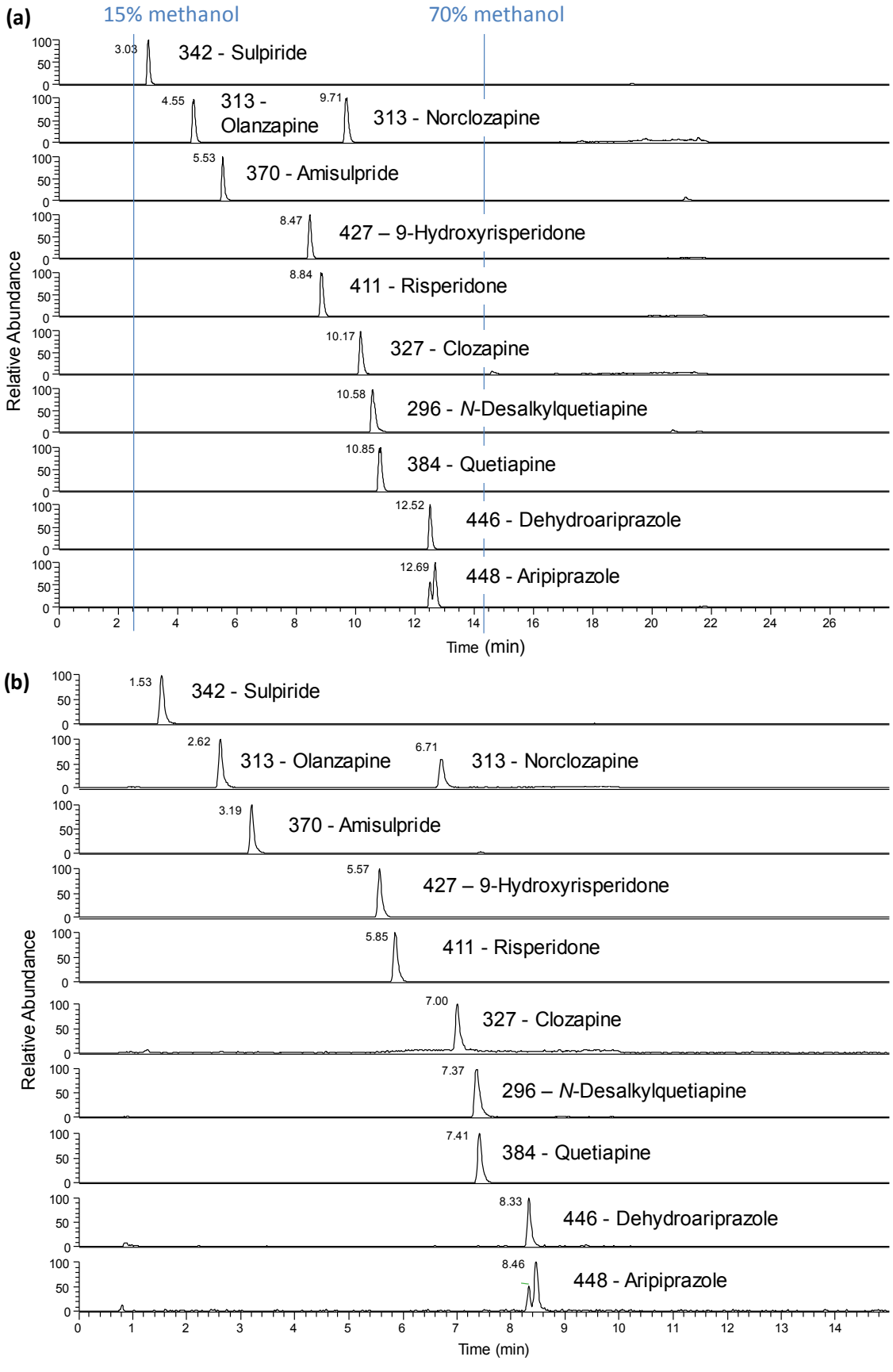
In-line UV is a useful way to detect analytes simultaneously within a single sample when the analytes display different physiochemical properties. The UV detector available used a deuterium lamp with maximum emitted wavelength of 370 nm. Both tartrazine and sunset yellow have maximum absorbance within the visible light range (tartrazine around 427 nm, sunset yellow in the region of 480-500 nm), therefore a non-maximal peak wavelength of 258 nm had to be selected to utilise the available UV lamp.

A test solution was prepared containing all analytes (antipsychotics as well as tartrazine and sunset yellow, 1 mg/L) and injected using a scouting gradient of 5 % to 100 % methanol-based eluent and full scan MS for the antipsychotics and UV for the dyes tested at both 370 nm and 258 nm. All other relevant analytes were retained and separated, and a 15-70 % methanol gradient enabled all analytes to elute in 9 minutes with a 5.8 minute re-equilibration step (Figure 3-2).

No response was detected for tartrazine either by UV (both wavelengths) or MS, suggesting that tartrazine may have eluted in the solvent front. Reversing the gradient direction gave the same results suggesting that tartrazine does not interact with the C18 column.

Simple spectrophotometry was therefore considered for tartrazine analysis.

Figure 3-2: Development of a reversed phase chromatographic method for analytes of interest.
MS conditions using full scan in Q3, 0.5 sec scan time, m/z 100-600 and extracted relevant ions. Mobile phase A = 99 % water, 1 % methanol with 0.1 % (v/v) formic acid; B = methanol with 0.1 % (v/v) formic acid.
(a) Gradient 5 % to 100 % B (21 min) to 5 % B (21.2 min) and equilibrate up to 28 min
(b) Gradient 15 % to 70 % B (9 min) to 15 % B (9.2 min) and equilibrate up to 15 min



3.3.3 Tartrazine by spectrophotometry

The commercial quantification method recommended measurement of the extinction coefficients for two wavelengths and calculation of the extinction difference (450 nm minus 520 nm; Greiner Bio-One, 2011). The wavelength response at 520 nm was used to establish the degree of cloudiness of the sample where proteins (e.g. mucin) and particulate matter non-specifically interfere, thereby leaving the specific wavelength result for 450 nm. The kit contained 5 calibrator solutions, 2 quality control solutions.

As a modification of the recommended analyser approach that could not be used due to the absence of a suitable automated analyser, a manual spectrophotometric method was designed where the samples were analysed in series by the first wavelength, recording the extinctions, and then changed to the second, and the results again recorded. An Excel spreadsheet was created containing a template of the calibrator values, the extinction difference calculated, and the results plotted to form a calibration curve (calculating the gradient, intercept and correlation) from which to calculate the results of samples analysed in U/mL (units of oral fluid per mL of collection fluid) as per the kit. Results were converted to give the percentage of oral fluid contained within the collection fluid (% oral fluid) by the result in U/mL multiplied by 100. This value could then be used to correct the analyte concentration within the collection fluid.

Although SGAs do not generally have an absorbance within the visible wavelength, potential interferences had to be ruled out. Blank oral fluid was collected as per the collection protocol from a drug-free volunteer and a portion set aside to verify the oral fluid content prior to addition of the analytes. A solution was made at 100 µg/L (all antipsychotics) using part of the volunteer sample, and analysed alongside the blank. Results were compared and there was no difference found in the calculated oral fluid content between the blank solutions and those with the added analytes, suggesting that as expected the presence of antipsychotics did not interfere with tartrazine quantification.

Blank buffer solution was provided by GBO that was equivalent to the extraction solution to be held in the mouth, but containing the preservative sodium azide, normally added at the final stage post-collection. In order to confirm this displayed the same spectrophotometric response as the solution prior to collection and the collection fluid following the complete protocol with sodium azide added, a comparison was undertaken. Oral fluid was collected from a volunteer,

however when spat into the beaker a portion was separated out so that it didn't contain sodium azide, and the remainder taken up as normal into the transfer tube containing sodium azide. A comparison between the directly analysed blank buffer solution and the volunteer samples with- and without-azide showed equivalent response of oral fluid content irrespective of the azide presence. The blank buffer solution was calculated to contain zero oral fluid content as expected (following dilution with deionised water to bring the tartrazine within the calibration range). All samples also all displayed the same pH (4-6) as measured by pH strips. The buffer solution could therefore be used as a blank for the creation of the calibration solutions if deemed appropriate by the recovery studies.

3.4 Method development for the antipsychotics

As tartrazine was not assayable by LC, development of the antipsychotic assay was undertaken using LLE-SCX.

3.4.1 Mass spectrometric conditions

Analyte transitions and MS conditions were taken from Fisher *et al*. (2013B) and Fisher *et al*. (2012B). Analyte tuning was undertaken for fluoxetine and norfluoxetine, and for the internal standards fluoxetine-D_6 and norfluoxetine-D_5. Product ions were selected based on the intensity of the response and the gases and temperature settings selected as per the other analytes within the published methods (Table 3.1).. Injection of test solutions containing each analyte (1 mg/L) showed no cross-talk and adequate sensitivity, therefore these transitions were added to the method (Table 3.1).

Upon starting the method assessment, poor reliability was identified for the new analytes, especially norfluoxetine. These analytes fragmented only to one major product ion within the range usually selected (>100 *m/z*), therefore in order to achieve 2 product ions a smaller than ideal ion was monitored. Investigation showed non-specific variability in the lower *m/z* product (between 30 and 44 *m/z*) and investigation of other transitions showed similar poor robustness or poor abundance; therefore a single product ion was selected for each of these analytes (at slightly higher collision energy) for the subsequent analysis.

Table 3.1: Mass spectrometric detection and analyte settings
Detection was by positive ion APCI. MS/MS settings: corona discharge current 4 µA; vaporiser and capillary temperatures 320 ºC and 300 °C respectively; auxiliary, sheath and ion sweep gas settings 5, 35, and 0 arbitrary units, respectively; scan width: 0.05 m/z; scan time: 40 ms; collision pressure (Ar): 1.5 mTorr

Analyte	**Precursor *m/z***	**Tube lens (V)**	**Product 1**		**Product 2**	
			m/z	**Collision energy (V)**	***m/z***	**Collision energy (V)**
N-Desalkylquetiapine	296.03	103	139.1	52	210.0	28
Norfluoxetine	296.06	84	30.6	20	134.1	5
	* 296.06	84	134.1	10	-	-
Norfluoxetine-D_5	301.10	83	32.5	38	139.2	5
	* 301.13	83	139.2	10	-	-
N-Desalkylquetiapine-D_8	303.99	105	182.9	37	209.8	28
Fluoxetine	310.07	85	44.5	12	148.1	5
	* 310.07	85	148.1	10	-	-
Norclozapine	313.04	90	192.1	38	270.0	21
Olanzapine	313.12	83	198.0	36	256.1	20
Olanzapine-D_3	316.04	87	198.0	40	256.0	21
Fluoxetine-D_6	316.10	84	44.2	12	154.2	6
	* 316.13	84	154.2	10	-	-
Norclozapine-D_8	321.20	93	192.1	39	275.1	23
Clozapine	327.09	86	192.1	40	270.1	20
Clozapine-D_8	335.14	90	192.0	43	275.0	23
O-Desalkylquetiapine	340.05	101	210.0	33	253.0	20
Sulpiride	342.10	86	112.2	24	214.0	30
Sulpiride-D_3	345.00	107	112.1	26	216.8	31
Amisulpride	370.11	84	195.6	39	242.0	25
Amisulpride-D_5	375.14	85	196.0	38	242.0	26
Quetiapine	384.07	100	221.1	34	253.0	21
Quetiapine-D_8	392.13	110	226.1	36	258.0	22
7-Hydroxyquetiapine	400.06	113	208.0	39	237.0	36
7-Hydroxyquetiapine-D_8	408.09	95	241.0	34	274.0	23
Risperidone	411.15	86	110.1	42	191.1	26
Risperidone-D_4	415.17	84	114.2	44	195.1	29
9-Hydroxyrisperidone	427.14	95	110.1	37	207.1	26
9-Hydroxyrisperidone-D_4	431.09	118	114.0	38	211.0	25
Dehydroaripiprazole	446.08	93	98.0	34	285.0	22
Aripiprazole	448.10	105	176.0	30	285.0	23
Dehydroaripiprazole-D_8	454.15	97	106.3	35	293.1	23
Aripiprazole-D_8	456.15	100	176.1	30	293.1	25

* Final settings indicated for fluoxetine, norfluoxetine and ISs.

3.4.2 Method development: plasma

All analytes of interest were not currently analysed within a single detection method (Fisher *et al*., 2013B; Fisher *et al*., 2012B), therefore the detection settings were combined as per section 3.4.1. Existing plasma calibration and IQC solutions for the SGAs and quetiapine metabolites were analysed on the combined method using the transitions as per Table 3.1 and extracted according to the published reports (summarised in Figure 3-3) . All results matched according to the nominal values from the individual methods; therefore the combined method was accepted for plasma analysis.

3.4.3 Optimising sample preparation for the GBO system

Use of LLE to extract the analytes from the sample matrix enables sample preparation to be very selective when optimised correctly. The existing plasma LLE extraction (Figure 3-3) uses 100 µL of 2 mol/L Tris solution at pH 10.6 to adjust the pH enabling optimum extraction of the analytes in question (two pH units above the pKa of most of the analytes).

The GBO collection solution buffered the collected oral fluid sample to approximately pH 4-6 with sodium citrate within the collection device. Therefore use of 2 mol/L Tris solution at pH 10.6 to extract the analytes from this acidic buffer solution was unlikely to modify the pH of the collection fluid to the desired basic pH without adding a very large volume of buffer. As such, stronger bases (higher pH) were investigated so that a small volume of basic solution could be added yet still buffer the solution to approximately pH 10-11. Use of too high a pH was avoided due to the risk of degradation of the analytes and metabolites.

Taking separate 1 mL portions of the buffer solution (initial pH 4-6), ammonium and sodium hydroxide (both 1 mol/L) were added to each portion and the pH tested. The results (Figure 3-3) showed that adding 50 µL ammonium hydroxide solution to 1 mL of buffer gave an appropriate extraction pH.

To confirm that the presence of oral fluid did not affect the pH reached when using the ammonium hydroxide solution, the experiment was repeated using blank oral fluid collected from a volunteer. Analogous results were obtained; hence this sample preparation method was further evaluated.

Figure 3-3: Summary of the plasma LLE sample extraction process.

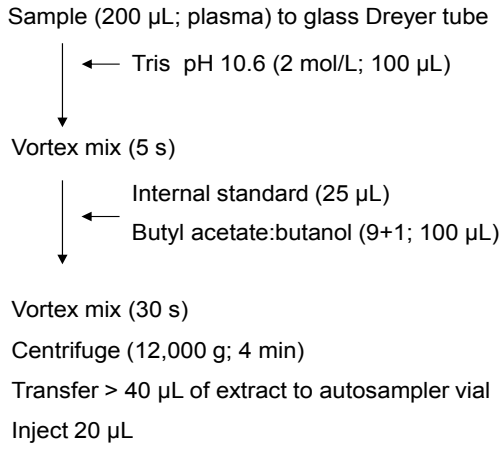
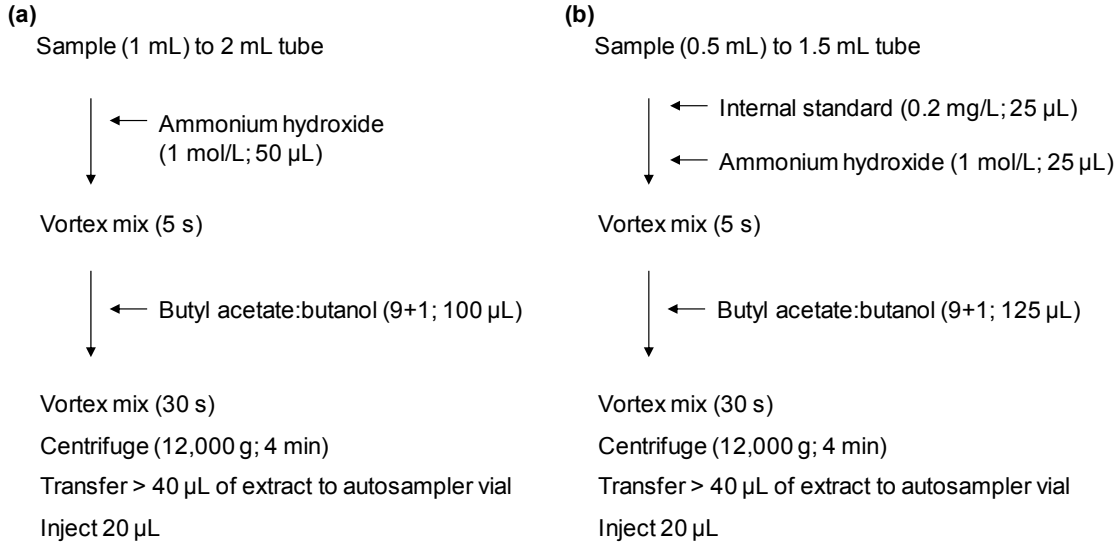


Table 3.2: Oral fluid sample pH adjustment testing results

Basic solution (1 mol/L)	Volume added to 1 mL buffer solution (µL)	pH achieved
Sodium hydroxide	25	pH 9-10
Sodium hydroxide	50	pH 13
Ammonium hydroxide	50	pH 10-11
Ammonium hydroxide	75	pH 11

Figure 3-4: Summary of the oral fluid LLE sample extraction process.
(a) Initial development; and (b) optimised method.



To ensure adequate analyte sensitivity, a sensitivity test solution of all analytes (10 µg/L) in blank GBO buffer solution was extracted using 50 µL of ammonium hydroxide (Figure 3-4a). Sensitivity was adequate, although a degree of emulsification was observed in some extracts. Due to the inconvenience of using screw cap 2 mL tubes for sample extraction, an alternative 1.5 mL flip-cap tube was investigated. A reduction in sample volume to 0.5 mL was therefore tested using 25 µL ammonium hydroxide solution and the volume of extraction solvent was increased to 125 µL to prevent emulsions from forming. The sensitivity test solution (10 µL, all analytes) was extracted again using the modified method (Figure 3-4b) using an internal standard solution (0.2 mg/L, all internal standards, in 0.1 mol/L hydrochloric acid). The results using the modified method showed good sensitivity and signal to noise ratio for all analytes and internal standards (Figure 3-5:a) and therefore this sample preparation method was adopted.

3.4.4 Optimising sample preparation for the Oral-Eze system

With sample preparation and extraction optimised for the GBO system, a similar protocol was followed to ensure the accurate assay of samples collected via the Oral-Eze system.

The collection solution for the Oral-Eze system was similar to that of the GBO system in that it was an acidic solution, therefore it was thought that a similar LLE method would be possible. As such, repeating the pH tests showed that use of 25 µL ammonium hydroxide solution (1 mol/L) added to 0.5 mL Oral-Eze buffer successfully increased the pH of the collection solutions to pH 10-11. Due to the dilution 1+2 of the oral fluid into collected buffer, the sensitivity experiments were tested at one-third the concentrations for the GBO samples, and sensitivity was adequate (Figure 3-5:b).

Figure 3-5: Results of the oral fluid sample extraction sensitivity tests.
Extraction of a test solution (20 µg/L group A analytes, 5 µg/L group B analytes, 2 µg/L group C analytes; analyte groups as per Table 3.3) using 0.5 mL sample volume as per the protocol.
(a) Test solution made in GBO buffer
(b) Test solution made in Oral-Eze buffer at one-third nominal concentrations

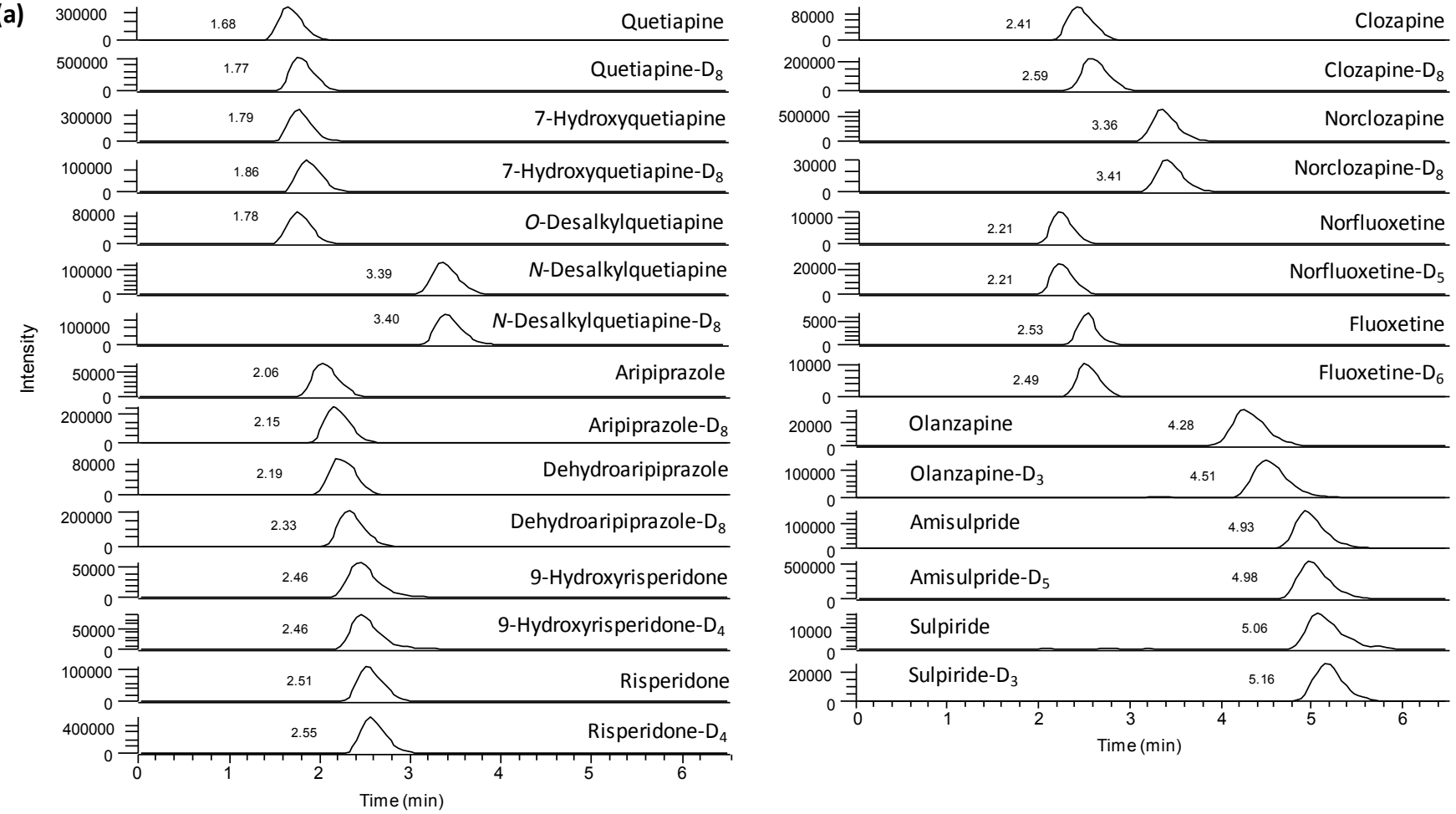
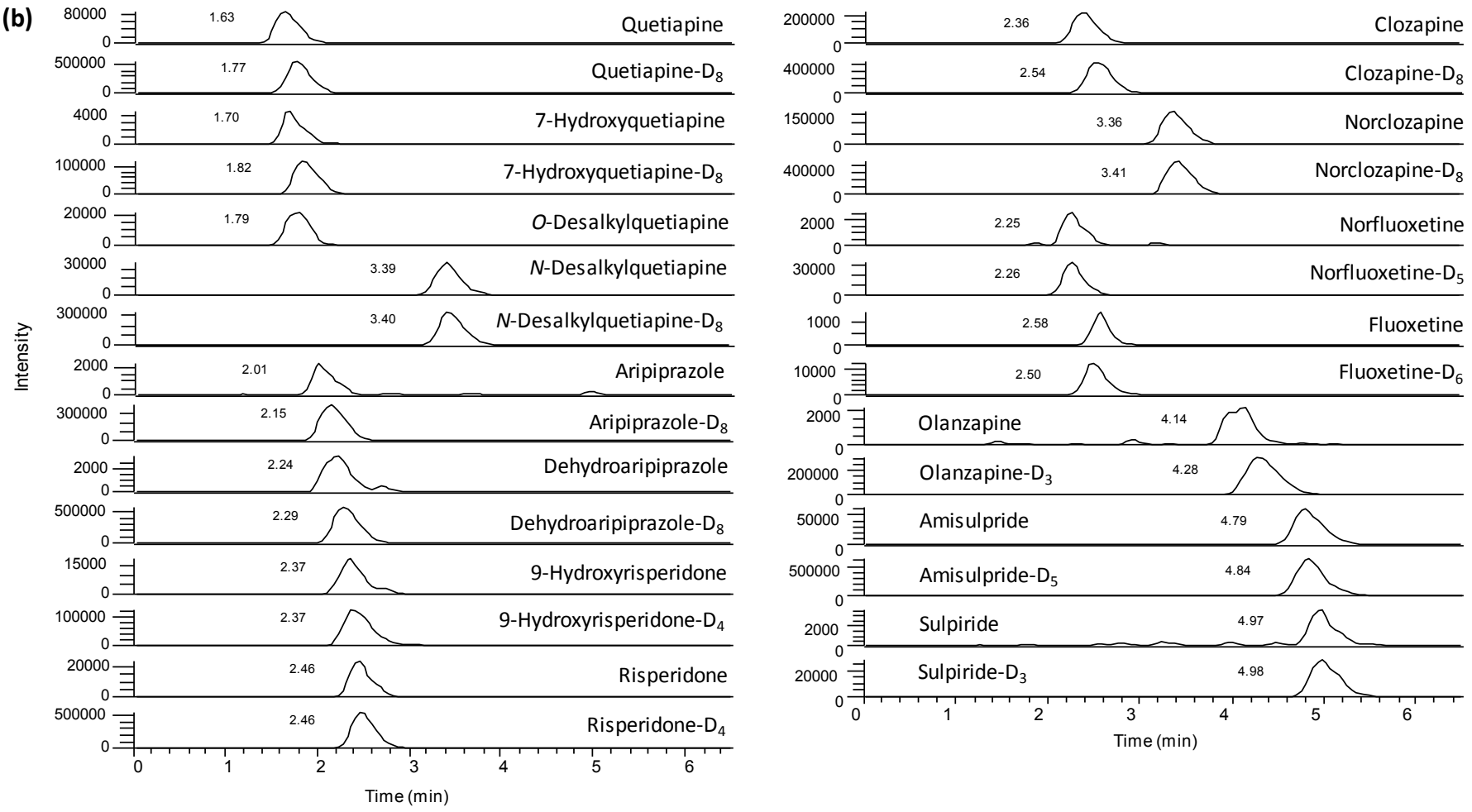


Figure 3-5: (cont.)



3.5 Method assessment

3.5.1 Method assessment protocol

Calibration (N = 7) and internal quality control (IQC; N = 3) solutions were prepared separately in combined groups with the analytes at 3 different concentrations depending on the expected analyte concentrations predicted from plasma concentrations (Table 3.3).

The antipsychotic methods were assessed based on the FDA guidelines for bioanalytical methods (FDA/CDER, 2001) including within- and between-batch accuracy and imprecision calculated from the IQC solutions, limit of detection (as calculated from dilution of calibrator 3), and linearity (as calculated from dilution of calibrator 7). Inaccuracy was defined as concentrations outside 20 % at the lower concentration (accuracy 80-120 %) and 15 % for the medium and high concentrations (accuracy 85-115 %). Imprecision was taken as a variation (relative standard deviation, RSD %) of greater than 15 % at all concentrations. Linearity and limit of detection were deemed acceptable when the measured concentration was within 15 % of the nominal concentration for each dilution. Carry-over was assessed by analysis of the highest calibrator solution and then the lowest calibrator (N = 3 in each case); the result from the first low concentration calibrator was then compared to the average concentration of the three low calibrators – a difference of < 15 % from the nominal concentration was deemed acceptable.

Matrix effects, extraction recovery, and process efficiency (overall recovery) were calculated for all analytes in all matrices using pre- and post-extraction addition to a defined analyte concentration (Matuszewski *et al*., 2003).

Initial GBO sample stability was assessed in a similar manner to that used previously (Fisher *et al*., 2013C), i.e. as a percentage of the response to that of a directly injected methanolic equivalent stored at -18 to -20 ºC for the same length of time. To assess long-term analyte stability, samples were prepared in each matrix at concentrations matching either IQC B or calibration solution 4 depending on the matrix type and validated at the time of preparation. Following storage at -18 to -20 ºC for 6 months, the samples were reanalysed and compared to the nominal concentration. Results with variation <15 % from nominal were considered to indicate analyte stability.

Table 3.3: Analyte concentrations. i) analyte groups and working solution concentrations; ii) calibration solution concentrations; and iii) IQC solution concentrations

i)

Group A (10 mg/L working solution)	**Group B (5 mg/L working solution)**
Clozapine	Amisulpride
Norclozapine	Aripiprazole
Group C (2 mg/L working solution)	Dehydroaripiprazole
Olanzapine	Fluoxetine
Risperidone	Norfluoxetine
9-Hydroxyrisperidone	Quetiapine
O-Desalkylquetiapine	*N*-Desalkylquetiapine
7-Hydroxyquetiapine	Sulpiride

ii)

Group	**Calibration solution concentration (µg/L)**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
A	5	20	50	200	500	1000	2000
B	2	5	20	50	150	400	800
C	1	2	5	20	50	100	200

iii)

Group	**IQC concentration (µg/L)**		
	A	**B**	**C**
A	30	300	900
B	6	75	300
C	3	30	90

Analyte recovery from the Oral-Eze pad was investigated by preparing a solution (100 µg/L all analytes) in blank oral fluid collected from two drug-free volunteers by the 'drool' technique. This solution (1 mL) was applied to Oral-Eze pads. Once the liquid was fully absorbed, the pads were separately immersed in Oral-Eze collection buffer (2 mL) for 1, 3, and 18 h (i.e. overnight), at 2-8 ºC prior to the fluid being separated from the pad in the normal way and stored in 2 mL polypropylene tubes at 2-8 ºC until analysed. The results were compared to those obtained from analysis of a 1+2 dilution of the 100 µg/L solution in Oral-Eze buffer.

3.5.2 Preliminary extraction recovery

To verify the suitability of the internal standards, and establish which matrix would be appropriate to prepare calibration standards, analyte recoveries were investigated. Recovery was established from the GBO oral fluid collection solution (100 µg/L all analytes including ISs, compared to a directly injected methanolic equivalent). The results (Table 3.4) are given as an overall percentage, and then given as a ratio to the corresponding IS to correct for compensation by the appropriate IS.

The recovery of some analytes, in particular sulpiride and 7-hydroxyquetiapine, were not corrected for by the IS used. Additional ISs (dehydroaripiprazole-D_8, *N*-desalkylquetiapine-D_8, 7-hydroxyquetipiapine-D_8, 9-hydroxyrisperidone-D_4 and sulpiride-D_3) were therefore purchased, and a test solution of each IS prepared in methanol for further investigation. A specific isotope-labelled IS was not available for *O*-desalkylquetiapine, however the preliminary experiment showed it was well compensated for by quetiapine-D_8.

All new ISs were tuned and the most suitable transitions incorporated into the instrument method as with the previous analytes (Table 3.1). Analyte recovery was retested, including new ISs, from a variety of different matrices in order to investigate potential matrices to use to prepare the calibration and IQC solutions. Since both collection systems rely upon an acidic buffer, the matrices tested were each manufacturer's blank buffer solution, a blank oral fluid sample collected using each collection system from a drug-free volunteer, deionised water, and 0.1 mol/L hydrochloric acid (Table 3.5).

Recoveries from each matrix were broadly comparable between the devices. However, there were some cases where recovery from the matrices was not equivalent, i.e. quetiapine and its metabolites, and sulpiride. The extraction recovery for the Oral-Eze samples was most comparable to that from deionised water, whereas for the GBO samples the recoveries were most comparable to that from 0.1 mol/L hydrochloric acid. As such, 0.1 mol/L hydrochloric acid and deionised water were used to prepare the calibration solutions for the GBO samples and for the Oral-Eze samples, respectively. The recoveries show that the blank matrices provided by each manufacturer gave equivalent results to the volunteer oral fluid samples.

Table 3.4: Extraction recovery (%) from GBO buffer compared to a methanolic equivalent, and corrected for IS

Analyte	**Recovery**	**Corrected for I.S**	
Amisulpride	85	*103*	Amisulpride-D_5
Sulpiride	22	**26**	Amisulpride-D_5
Amisulpride-D_5	83	-	
Aripiprazole	105	*100*	Aripiprazole-D_8
Dehydroaripiprazole	112	*107*	Aripiprazole-D_8
Aripiprazole-D_8	105	-	
Clozapine	116	*101*	Clozapine-D_8
Clozapine-D_8	115	-	
Norclozapine	128	*108*	Norclozapine-D_8
Norclozapine-D_8	118	-	
Fluoxetine	106	*106*	Fluoxetine-D_6
Fluoxetine-D_6	99	-	
Norfluoxetine	117	118	Norfluoxetine-D_5
Norfluoxetine-D_5	99	-	
Olanzapine	123	*101*	Olanzapine-D_3
Olanzapine-D_3	121	-	
Quetiapine	116	*101*	Quetiapine-D_8
N-Desalkylquetiapine	130	*113*	Quetiapine-D_8
O-Desalkylquetiapine	116	*100*	Quetiapine-D_8
7-Hydroxyquetiapine	80	**69**	Quetiapine-D_8
Quetiapine-D_8	115	-	
Risperidone	120	*104*	Risperidone-D_4
9-Hydroxyrisperidone	114	*99*	Risperidone-D_4
Risperidone-D_4	115	-	

Table 3.5: Extraction recovery (%) from the buffer solutions compared to methanolic equivalent

Analyte	**0.1 mol/L hydrochloric acid**	**GBO buffer**	**GBO sample**	**Oral-eze buffer**	**Oral-eze sample**	**Deionised water**
Amisulpride	64	71	69	69	71	72
Amisulpride-D_5	*61*	*63*	*66*	*68*	*65*	*65*
Aripiprazole	102	106	104	105	97	103
Aripiprazole-D_8	*107*	*107*	*101*	*104*	*101*	*104*
Dehydroaripiprazole	104	108	104	107	100	101
Dehydroaripiprazole-D_8	*107*	*107*	*101*	*104*	*101*	*104*
Clozapine	105	109	111	114	107	108
Clozapine-D_8	*107*	*113*	*115*	*117*	*110*	*108*
Norclozapine	87	92	90	94	88	91
Norclozapine-D_8	*88*	*91*	*91*	*93*	*88*	*91*
Fluoxetine	83	88	81	89	83	85
Fluoxetine-D_6	*86*	*86*	*76*	*81*	*80*	*84*
Norfluoxetine	111	99	110	69	67	67
Norfluoxetine-D_5	*73*	*76*	*70*	*83*	*74*	*76*
Olanzapine	103	113	113	106	85	106
Olanzapine-D_3	*101*	*107*	*112*	*105*	*83*	*102*
Quetiapine	105	115	117	77	90	111
O-Desalkylquetiapine	105	109	114	88	91	107
Quetiapine-D_8	*105*	*109*	*114*	*93*	*93*	*106*
N-Desalkylquetiapine	99	106	102	98	101	106
N-Desalkyquetiapine-D_8	*94*	*98*	*95*	*97*	*93*	*96*
7-Hydroxyquetiapine	83	96	97	42	45	39
7-Hydroxyquetiapine-D_8	*80*	*95*	*93*	*44*	*44*	*37*
Risperidone	103	109	111	107	102	102
Risperidone-D_4	*99*	*106*	*111*	*107*	*100*	*105*
9-Hydroxyrisperidone	95	103	107	97	95	99
9-Hydroxyrisperidone-D_4	*93*	*100*	*109*	*99*	*94*	*99*
Sulpiride	21	28	24	10	11	8
Sulpiride-D_3	*21*	*27*	*23*	*10*	*10*	*7*

3.5.3 Preparation of calibration and internal quality control solutions

Due to the large number of analytes, intermediary working solutions were prepared for the analytes grouped at three different concentrations to match the predicted concentrations of the analytes expected in samples based on the plasma analyte concentrations (Table 3.3). These intermediary solutions were prepared, and the calibration and IQC solutions independently produced, by pipetting appropriate volumes of the stock solutions into volumetric glassware and making up to volume with the respective matrices before being separated into smaller portions and stored at -18 to -20 ºC until used. Only STD 1-4 and IQC A and B (Table 3.3) could be produced for norfluoxetine due to the limited availability of the reference compound leading to a finite volume of solution being available.

In line with the recovery experiments, the calibration solutions for the GBO system were prepared in 0.1 mol/L hydrochloric acid and the IQC solutions in the GBO buffer; for the Oral-Eze system calibration solutions were prepared in deionised water with the IQC solutions being prepared in the Oral-Eze buffer. Due to the 1+2 dilution of patient oral fluid samples in the Oral-Eze collection system the calibration and IQC solutions were prepared at one-third of nominal to reflect the patient samples and make the analytical measurement as comparable as possible with the clinical samples and the calibration and IQC solutions. Plasma calibration and IQC solutions at the same concentrations were prepared in dipotassium EDTA plasma (Table 3.3).

Full calibration curves were extracted and nominal concentrations verified for all IQCs for the plasma, Oral-Eze and GBO methods.

3.5.4 Analyte calibration

Calibration graphs for all analytes were linear within the calibration range, with R^2 >0.9 for all methods and analytes (Figure 3-6).

Figure 3-6: Examples of analyte calibration curves

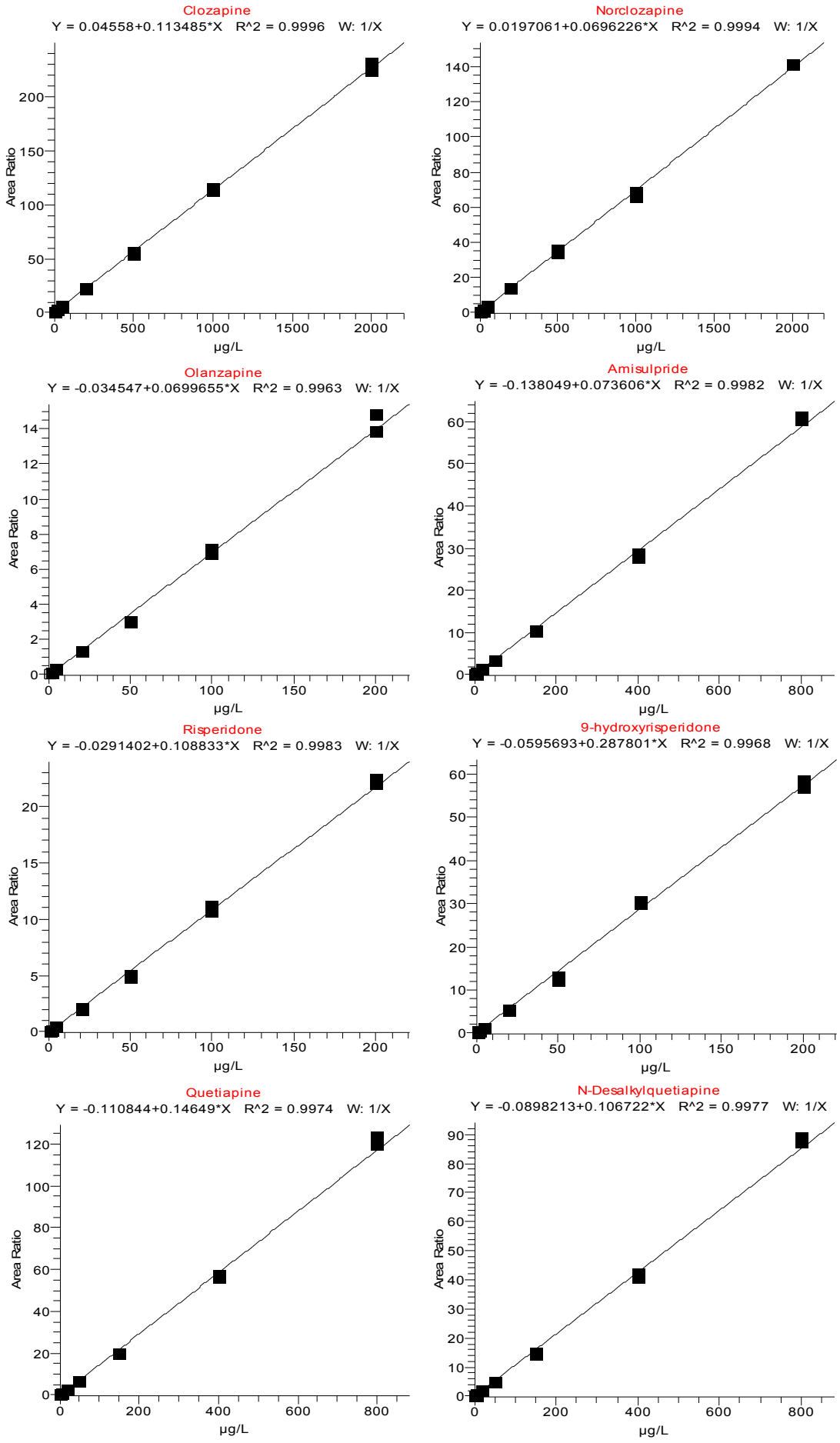
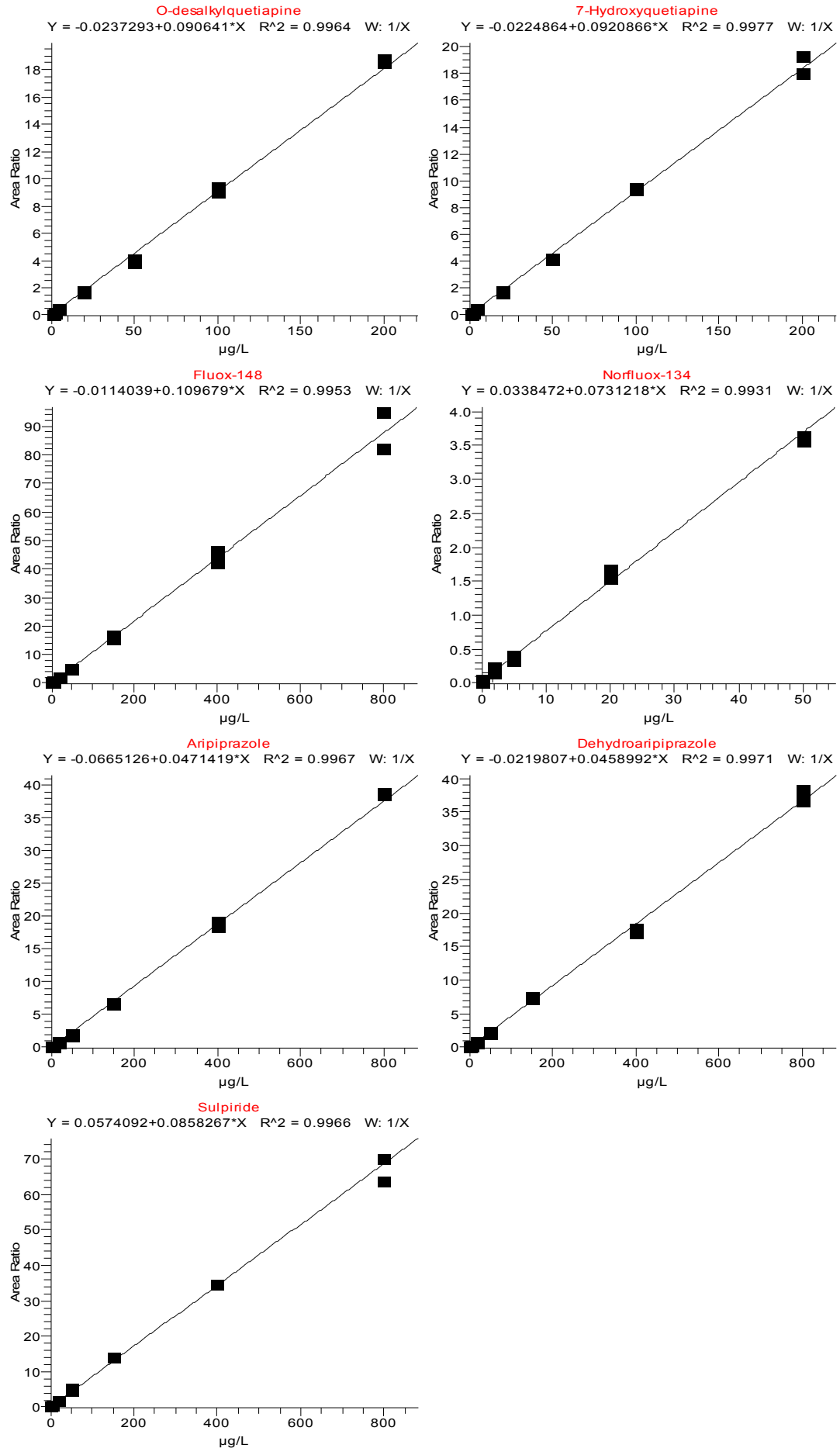


Figure 3-6 (cont.)



3.5.5 Limit of detection, linearity and carryover

Carryover was assessed for all analytes for all methods, and was below 15 % for all analytes. Limits of detection are given in Table 3.6, and show that the assigned limits of detection were generally higher for the Oral-Eze system method as would be expected due to the one-third dilution of the calibration and IQC solutions.

Table 3.6: Limits of detection for each analytical method

Analyte	**Limit of detection (µg/L)**	
	GBO system method	**Oral-Eze system method**
Amisulpride	5	5
Aripiprazole	2	5
Dehydroaripiprazole	2	5
Clozapine	5	10
Norclozapine	5	10
Fluoxetine	2	5
Norfluoxetine	2	5
Olanzapine	2	5
Quetiapine	2	5
N-Desalkylquetiapine	2	5
O-Desalkylquetiapine	2	2
7-Hydroxyquetiapine	2	2
Risperidone	1	2
9-Hydroxyrisperidone	1	2
Sulpiride	2	5

3.5.6 Within- and between-batch accuracy and precision

3.5.6.1 GBO device

Results for the accuracy and precision studies for the GBO system method are given within-batch (Table 3.7) and between-batch (Table 3.8). Full data are given in Appendix B.

Aripiprazole and dehydroaripiprazole displayed significant inaccuracy (both within- and between-batch), possibly caused by the analyte solubility and stability issues that have been described previously (Fisher *et al.*, 2013B), or due to an error with production of the calibrators and IQCs. These issues were not further investigated since these analytes played not further part in the clinical study. Within-batch results were generally precise, as indicated by the low RSD%, however between-batch the variation became much higher.

3.5.6.2 Oral-Eze device

Results for the accuracy and precision studies for the Oral-Eze system method are given within-batch (Table 3.9) and between-batch (Table 3.10). Full data are given in Appendix B.

As with the GBO system method there was a large inaccuracy observed both between- and within-batch for aripiprazole and dehydroaripiprazole, and no further investigations were carried out. Fluoxetine and norfluoxetine displayed some within-batch imprecision at low concentration as with the GBO system method. This was taken as acceptable since clinically the difference in result obtained would have little meaning, and these analytes did not play a major part in the further study.

For the Oral-Eze method there was a large degree of inaccuracy for a number of the analytes, especially at low concentration. This could be related to the lower assay sensitivity (Figure 3-5:) due to the one-third dilution of the solutions compared to nominal to account for the dilution of oral fluid samples into the collection buffer.

Table 3.7: Within-batch accuracy and precision for the GBO system method.
Results imprecise or inaccurate highlighted in bold. N = 5

Nominal [analyte] (µg/L)		Mean (µg/L)	RSD (%)	Accuracy (%)
Amisulpride	6	7	2.3	111
	75	70	4.6	93
	300	282	3.8	94
Aripiprazole	6	4	14.7	***63***
	75	34	2.0	***45***
	300	218	1.7	***73***
Dehydroaripiprazole	6	3	***21.0***	***49***
	75	32	2.1	***43***
	300	223	3.4	***74***
Clozapine	60	51	2.8	85
	300	275	1.6	92
	900	866	3.5	96
Norclozapine	60	50	4.3	83
	300	272	2.5	91
	900	836	0.9	93
Fluoxetine	6	6	14.5	100
	75	76	10.3	102
	300	291	14.4	97
Norfluoxetine	6	6	10.5	100
	75	80	5.2	107
Olanzapine	3	3	4.0	90
	30	26	1.1	85
	90	79	3.6	88
Quetiapine	6	7	6.1	110
	75	66	1.9	88
	300	278	2.1	93
N-Desalkylquetiapine	6	7	8.0	111
	75	68	4.9	90
	300	271	5.1	90
O-Desalkylquetiapine	3	3	3.2	104
	30	30	2.9	100
	90	91	1.4	101
7-Hydroxyquetiapine	3	3	7.1	96
	30	29	2.7	98
	90	89	3.5	98
Risperidone	3	3	5.3	94
	30	29	2.1	97
	90	88	1.7	97
9-Hydroxyrisperidone	3	3	11.7	101
	30	29	2.9	97
	90	88	4.4	98
Sulpiride	6	6	14.7	105
	75	85	3.3	114
	300	317	5.6	106

Table 3.8: Between-batch accuracy and precision for the GBO system method.
Results imprecise or inaccurate highlighted in bold. N = 4

Nominal [analyte] (µg/L)		Mean (µg/L)	RSD (%)	Accuracy (%)
Amisulpride	6	7	6.3	116
	75	71	3.3	94
	300	284	2.1	95
Aripiprazole	6	3	***19.8***	***54***
	75	29	***17.1***	***39***
	300	213	7.2	***71***
Dehydroaripiprazole	6	3	9.1	***44***
	75	29	***19.2***	***38***
	300	222	5.4	***74***
Clozapine	60	50	2.8	83
	300	285	8.8	95
	900	851	2.6	95
Norclozapine	60	50	3.8	84
	300	273	3.9	91
	900	850	3.6	94
Fluoxetine	6	6	8.0	99
	75	75	13.5	100
	300	290	1.4	97
Norfluoxetine	6	7	11.9	111
	75	79	11.3	105
Olanzapine	3	3	5.9	92
	30	26	6.9	86
	90	79	4.9	87
Quetiapine	6	6	3.1	105
	75	66	1.7	89
	300	276	2.0	92
N-Desalkylquetiapine	6	6	3.4	106
	75	67	3.9	89
	300	277	2.2	92
O-Desalkylquetiapine	3	3	2.3	102
	30	30	1.5	101
	90	90	1.5	100
7-Hydroxyquetiapine	3	3	1.4	95
	30	29	1.8	97
	90	92	13.2	102
Risperidone	3	3	3.2	91
	30	28	3.6	94
	90	86	2.4	96
9-Hydroxyrisperidone	3	3	2.5	98
	30	30	4.6	99
	90	90	5.8	100
Sulpiride	6	6	5.9	107
	75	85	12.6	114
	300	326	3.3	109

Table 3.9: Within-batch accuracy and precision for the Oral-Eze system method.
Results imprecise or inaccurate highlighted in bold. N = 5

Nominal [analyte] (µg/L)		Mean (µg/L)	RSD (%)	Accuracy (%)
Amisulpride	6	7	4.9	113
	75	73	2.5	97
	300	299	2.8	100
Aripiprazole	6	11	3.7	***187***
	75	85	3.3	114
	300	343	4.4	114
Dehydroaripiprazole	6	12	2.3	***199***
	75	103	3.6	***137***
	300	404	4.8	***135***
Clozapine	60	69	2.2	115
	300	290	3.1	97
	900	925	1.6	103
Norclozapine	60	68	2.4	113
	300	273	3.7	91
	900	898	3.2	100
Fluoxetine	6	7	***17.1***	115
	75	68	13.9	91
	300	307	9.5	102
Norfluoxetine	6	8	***16.8***	***140***
	75	77	9.4	102
Olanzapine	3	4	3.8	***142***
	30	33	3.5	109
	90	99	2.9	110
Quetiapine	6	6	5.2	97
	75	55	2.5	***73***
	300	225	2.5	***75***
N-Desalkylquetiapine	6	8	7.0	***133***
	75	80	2.3	106
	300	337	4.8	112
O-Desalkylquetiapine	3	3	8.2	101
	30	29	3.4	97
	90	85	2.1	94
7-Hydroxyquetiapine	3	3	12.6	90
	30	24	5.2	***80***
	90	72	7.0	***80***
Risperidone	3	4	4.8	***124***
	30	31	1.3	105
	90	97	4.6	108
9-Hydroxyrisperidone	3	3	6.5	107
	30	32	5.0	107
	90	98	5.2	109
Sulpiride	6	6	14.6	107
	75	87	8.9	***116***
	300	347	8.3	***116***

Table 3.10: Between-batch accuracy and precision for the Oral-Eze system method.
Results imprecise or inaccurate highlighted in bold. N = 4

Nominal [analyte] (µg/L)		Mean (µg/L)	RSD (%)	Accuracy (%)
Amisulpride	6	7	3.7	116
	75	74	2.2	99
	300	305	2.1	102
Aripiprazole	6	11	3.2	***189***
	75	90	4.4	***120***
	300	357	3.5	***119***
Dehydroaripiprazole	6	12	3.5	***200***
	75	106	4.6	***142***
	300	411	4.0	***137***
Clozapine	60	71	2.1	118
	300	290	2.4	97
	900	935	1.9	104
Norclozapine	60	66	8.4	111
	300	264	10.7	88
	900	847	11.7	94
Fluoxetine	6	7	5.9	112
	75	77	14.2	102
	300	304	8.8	101
Norfluoxetine	6	9	5.4	***150***
	75	72	11.8	96
Olanzapine	3	6	***31.0***	***187***
	30	34	6.1	113
	90	99	3.2	110
Quetiapine	6	6	5.7	105
	75	55	3.8	***74***
	300	228	3.2	***76***
N-Desalkylquetiapine	6	9	7.0	***143***
	75	85	6.1	113
	300	356	4.4	119
O-Desalkylquetiapine	3	3	4.4	100
	30	27	9.4	89
	90	81	5.6	90
7-Hydroxyquetiapine	3	3	6.1	85
	30	24	3.3	***81***
	90	74	5.4	***82***
Risperidone	3	4	4.0	***127***
	30	32	2.1	107
	90	97	1.4	107
9-Hydroxyrisperidone	3	4	11.7	***123***
	30	33	3.2	110
	90	104	7.7	***116***
Sulpiride	6	7	9.6	114
	75	90	7.5	***120***
	300	342	6.2	114

3.5.6.3 Plasma

The method used was the same as that previously presented (Fisher *et al*., 2013B; Fisher *et al*., 2012B), except that fluoxetine and norfluoxetine were added (Table 3.11). Accuracy was acceptable for all analytes meeting the published criteria, as well as for the added analytes, although there was some imprecision observed within these new analytes, especially at lower concentration.

3.5.7 Matrix effects, extraction recovery and process efficiency

As per the method first suggested by Matuszewski *et al*. (2003), matrix effects (Table 3.12), extraction recovery (Table 3.13) and process efficiency (overall recovery; Table 3.14) were calculated for all analytes in all matrices, and then displayed as corrected for IS (Table 3.15). The majority of the variation in process efficiency was attributable to extraction recovery (Table 3.13).

Table 3.11: Plasma method fluoxetine and norfluoxetine accuracy and precision.
Results imprecise or inaccurate highlighted in bold. (N = 4)
(A) Within-assay; (B) Between-assay

(A) Nominal [analyte] (µg/L)		**Mean (µg/L)**	**RSD (%)**	**Accuracy (%)**
Fluoxetine	6	6	**19.4**	107
	75	77	14.4	102
	300	305	14.2	102
Norfluoxetine	6	6	10.4	107
	75	68	12.9	91

(B) Nominal [analyte] (µg/L)		**Mean (µg/L)**	**RSD (%)**	**Accuracy (%)**
Fluoxetine	6	6	14.3	101
	75	74	6.6	99
	300	298	3.4	99
Norfluoxetine	6	6	5.6	102
	75	76	12.6	102

Table 3.12: Matrix effects of all analytes from different matrices (%; N = 4 in each case).

Analyte	0.1 mol/L hydrochloric acid	GBO buffer	GBO sample A	GBO sample B	Deionised water	Oral-Eze buffer	Oral-Eze sample A	Oral-Eze sample B	Plasma
Amisulpride	106	102	107	122	104	107	106	122	108
Amisulpride-D_5	*90*	*105*	*113*	*120*	*101*	*104*	*113*	*120*	*93*
Aripiprazole	100	99	131	112	95	90	131	112	130
Aripiprazole-D_8	*102*	*102*	*111*	*114*	*101*	*113*	*111*	*114*	*110*
Dehydroaripiprazole	96	97	106	110	93	87	95	110	128
Dehydroaripiprazole-D_8	*82*	*102*	*111*	*114*	*101*	*113*	*111*	*114*	*110*
Clozapine	90	91	98	104	96	83	98	104	121
Clozapine-D_8	*84*	*105*	*109*	*115*	*100*	*113*	*109*	*115*	*118*
Norclozapine	109	94	102	106	96	82	102	106	108
Norclozapine-D_8	*103*	*102*	*111*	*113*	*100*	*108*	*111*	*113*	*99*
Fluoxetine	106	92	101	116	114	99	101	116	100
Norfluoxetine	103	93	106	101	105	96	94	80	85
Norfluoxetine-D_5	*110*	*112*	*101*	*114*	*93*	*102*	*101*	*114*	*104*
Olanzapine	100	109	103	108	91	99	103	108	118
Olanzapine-D_8	*109*	*107*	*124*	*126*	*107*	*123*	*124*	*126*	*106*
Quetiapine	98	100	106	114	93	100	99	114	101
O-Desalkylquetiapine	105	103	114	118	99	108	114	118	128
Quetiapine-D_8	*105*	*104*	*113*	*118*	*101*	*102*	*113*	*118*	*105*
N-Desalkylquetiapine	102	103	108	121	97	98	107	121	129
N-Desalkylquetiapine-D_8	*104*	*102*	*115*	*124*	*102*	*93*	*115*	*124*	*100*
7-Hydroxyquetiapine	108	78	113	121	99	104	113	121	100
7-Hydroxyquetiapine-D_8	*95*	*103*	*112*	*118*	*100*	*95*	*112*	*118*	*78*
Risperidone	101	102	106	108	92	114	106	108	115
Risperidone-D_4	*85*	*104*	*115*	*118*	*103*	*123*	*115*	*118*	*98*
9-Hydroxyrisperidone	98	102	103	109	90	105	103	109	102
9-Hydroxyrisperidone-D_4	*83*	*104*	*113*	*97*	*98*	*87*	*113*	*122*	*106*
Sulpiride	94	99	104	106	89	103	129	106	130
Sulpiride-D_3	*81*	*101*	*117*	*125*	*99*	*113*	*117*	*125*	*110*

Table 3.13: Extraction recovery of all analytes from different matrices (%; N = 4 in each case).

Analyte	0.1 mol/L hydrochloric acid	GBO buffer	GBO sample A	GBO sample B	Deionised water	Oral-Eze buffer	Oral-Eze sample A	Oral-Eze sample B	Plasma
Amisulpride	73	73	80	75	79	55	62	57	64
Amisulpride-D_5	*72*	*73*	*70*	*69*	*78*	*61*	*53*	*52*	*60*
Aripiprazole	108	96	73	91	95	78	51	66	70
Aripiprazole-D_8	*101*	*103*	*95*	*95*	*107*	*69*	*64*	*65*	*64*
Dehydroaripiprazole	112	97	95	92	85	79	75	69	70
Dehydroaripiprazole-D_8	*131*	*103*	*95*	*95*	*107*	*69*	*64*	*65*	*64*
Clozapine	121	110	108	108	103	80	71	71	78
Clozapine-D_8	*136*	*102*	*99*	*102*	*112*	*66*	*69*	*67*	*71*
Norclozapine	105	109	106	106	100	80	68	74	69
Norclozapine-D_8	*105*	*109*	*97*	*101*	*108*	*75*	*83*	*86*	*64*
Fluoxetine	101	108	98	100	95	95	103	93	82
Norfluoxetine	108	102	90	109	83	57	47	60	59
Norfluoxetine-D_5	*96*	*96*	*99*	*98*	*110*	*50*	*59*	*53*	*51*
Olanzapine	112	99	94	96	65	76	72	76	82
Olanzapine-D_8	*109*	*102*	*98*	*98*	*108*	*64*	*66*	*72*	*84*
Quetiapine	111	100	103	100	116	36	49	48	100
O-Desalkylquetiapine	104	97	94	97	105	38	42	48	81
Quetiapine-D_8	*109*	*103*	*96*	*97*	*112*	*63*	*54*	*54*	*79*
N-Desalkylquetiapine	113	103	101	99	111	65	70	67	66
N-Desalkylquetiapine-D_8	*106*	*105*	*96*	*102*	*111*	*53*	*50*	*46*	*70*
7-Hydroxyquetiapine	64	75	44	55	31	11	12	9	86
7-Hydroxyquetiapine-D_8	*74*	*58*	*44*	*56*	*30*	*15*	*12*	*11*	*96*
Risperidone	106	95	94	97	114	65	66	70	84
Risperidone-D_4	*129*	*101*	*97*	*99*	*109*	*63*	*69*	*68*	*84*
9-Hydroxyrisperidone	101	90	89	90	112	61	60	62	89
9-Hydroxyrisperidone-D_4	*124*	*95*	*89*	*101*	*106*	*60*	*48*	*47*	*76*
Sulpiride	21	17	10	16	7	4	3	4	24
Sulpiride-D_3	*23*	*16*	*11*	*15*	*6*	*3*	*3*	*3*	*22*

Table 3.14: Process efficiency of all analytes from different matrices (%; N = 4 in each case).

Analyte	0.1 mol/L hydrochloric acid	GBO buffer	GBO sample A	GBO sample B	Deionised water	Oral-Eze buffer	Oral-Eze sample A	Oral-Eze sample B	Plasma
Amisulpride	77	74	85	92	82	59	67	70	70
Amisulpride-D_5	*74*	*76*	*79*	*83*	*79*	*62*	*60*	*63*	*55*
Aripiprazole	108	94	95	102	90	70	67	74	90
Aripiprazole-D_8	*108*	*105*	*105*	*109*	*108*	*78*	*71*	*75*	*71*
Dehydroaripiprazole	108	94	94	101	79	69	70	76	89
Dehydroaripiprazole-D_8	*108*	*105*	*105*	*109*	*108*	*78*	*71*	*75*	*71*
Clozapine	108	100	106	113	99	66	70	75	95
Clozapine-D_8	*114*	*107*	*108*	*117*	*112*	*74*	*75*	*77*	*83*
Norclozapine	111	102	108	113	97	66	69	78	75
Norclozapine-D_8	*112*	*111*	*107*	*114*	*108*	*91*	*92*	*97*	*63*
Fluoxetine	107	99	99	116	108	94	104	107	82
Norfluoxetine	111	95	96	111	87	55	44	48	50
Norfluoxetine-D_5	*106*	*108*	*100*	*112*	*102*	*51*	*60*	*61*	*53*
Olanzapine	112	104	97	103	79	80	74	82	96
Olanzapine-D_8	*114*	*109*	*122*	*123*	*115*	*79*	*81*	*91*	*87*
Quetiapine	109	100	109	115	108	37	49	55	101
O-Desalkylquetiapine	109	101	107	114	104	41	48	57	104
Quetiapine-D_8	*109*	*107*	*108*	*115*	*113*	*53*	*61*	*64*	*83*
N-Desalkylquetiapine	115	106	115	120	107	64	72	81	85
N-Desalkylquetiapine-D_8	*115*	*108*	*111*	*126*	*113*	*47*	*58*	*57*	*70*
7-Hydroxyquetiapine	69	58	50	66	30	9	13	11	89
7-Hydroxyquetiapine-D_8	*71*	*60*	*49*	*66*	*30*	*11*	*13*	*12*	*74*
Risperidone	107	97	99	105	105	74	70	76	97
Risperidone-D_4	*110*	*105*	*111*	*117*	*112*	*78*	*79*	*80*	*82*
9-Hydroxyrisperidone	98	92	91	98	101	64	61	68	90
9-Hydroxyrisperidone-D_4	*102*	*99*	*100*	*112*	*104*	*52*	*54*	*57*	*78*
Sulpiride	20	17	11	17	6	4	4	4	31
Sulpiride-D_3	*19*	*16*	*13*	*18*	*6*	*3*	*4*	*3*	*25*

Table 3.15: Process efficiency corrected for internal standard of all analytes from different matrices (%; N = 4 in each case).

Analyte	0.1 mol/L hydrochloric acid	GBO buffer	GBO sample A	GBO sample B	Deionised water	Oral-Eze buffer	Oral-Eze sample A	Oral-Eze sample B	Plasma
Amisulpride	104	98	108	111	103	94	111	111	126
Aripiprazole	100	90	91	94	83	89	94	99	127
Dehydroaripiprazole	100	90	90	93	73	88	98	101	126
Clozapine	95	94	98	97	88	89	93	97	113
Norclozapine	99	92	101	99	89	72	75	81	118
Fluoxetine	107	99	99	116	108	94	104	107	82
Norfluoxetine	104	88	96	99	85	107	73	79	95
Olanzapine	99	95	80	84	52	101	91	90	111
Quetiapine	100	93	100	100	95	70	80	85	122
N-Desalkylquetiapine	100	99	104	96	95	135	124	143	122
O-Desalkylquetiapine	100	94	98	100	92	76	78	89	125
7-Hydroxyquetiapine	98	98	101	101	102	82	99	91	120
Risperidone	97	93	90	90	93	95	88	95	119
9-Hydroxyrisperidone	96	93	91	88	97	123	115	119	116
Sulpiride	104	101	87	90	97	120	115	114	128

3.5.8 Analyte recovery from the Oral-Eze pad

The Oral-Eze device relies on adsorption of the oral fluid onto a pad and then equilibration of analytes from the pad into the collection buffer to a consistent degree, hence the impact of analyte adsorption onto the pad and equilibration time was considered.

A solution was prepared (100 µg/L, all analytes) in blank oral fluid collected by the drool technique (left to pool in the mouth) from drug-free volunteers. This solution was applied to the pad (1 mL) until all liquid was absorbed, and the pad was then submerged in the buffer for varying amounts of time before the pad was plunged down and the collection fluid separated into 2 mL tubes. These solutions were extracted and analysed, and the results compared to an extract from a sample where 1 mL of the solution was applied directly into the buffer without a pad. To minimise the impact of variation in equilibration time, and as per personal guidance from the Thermo technical specialist, it was decided that leaving the pad in the buffer overnight should be tested. Results (Table 3.16) showed that storage of the pad in the buffer solution enabled the analytes to equilibrate during storage since the analytes recoveries increased overnight (18 hours) compared to either 1 hour, or 3 hours. In addition, the results from oral fluid from two different sources were broadly in agreement, suggesting that whilst it was possible that there could be some variation dependent on the features of each individuals oral fluid (i.e. viscosity, pH etc), these effects were minimised by the dilution into the buffer solution.

The clinical samples were therefore stored in the refrigerator overnight, and then the fluid was plunged from the pad and the collected fluid stored in 2 mL polypropylene tubes at -18 to -20 ºC until analysed.

Due to the continued ongoing adsorption of analytes onto the pad even after equilibration, as indicated by the poor recovery of some analytes, clinical results from the analysis of samples collected using this system were corrected by the factor determined as the overall recovery in order to compensate for the fact the that calibration solutions did not undergo such losses. As such, the results were divided by the overall overnight recoveries described in Table 3.16 – for clarity the division factors are given in Table 3.17.

Table 3.16: Recovery of the analytes (%) off the pad when stored in the buffer prior to being plunged off for varying times compared to a solution not applied to the pad. Overnight was 18 hours. (N=6)

Analyte	1 Hour recovery (mean ± SD)			3 Hour recovery (mean ± SD)			Overnight recovery (mean ± SD)		
	Source 1	Source 2	Overall	Source 1	Source 2	Overall	Source 1	Source 2	Overall
Amisulpride	78 ± 4.5	73 ± 4.1	75 ± 4.8	84 ± 3.3	80 ± 5.2	82 ± 4.8	83 ± 4.1	83 ± 3.8	83 ± 3.8
Aripiprazole	36 ± 3.6	31 ± 2.4	34 ± 3.8	35 ± 3.4	32 ± 2.0	33 ± 3.1	38 ± 4.6	42 ± 3.9	40 ± 4.6
Dehydroaripiprazole	35 ± 4.5	32 ± 4.5	34 ± 4.5	35 ± 2.5	33 ± 1.8	34 ± 2.3	36 ± 5.1	41 ± 2.5	38 ± 4.4
Clozapine	52 ± 2.2	50 ± 4.5	51 ± 3.6	59 ± 2.1	62 ± 5.5	61 ± 4.4	63 ± 5.8	75 ± 4.3	69 ± 8.3
Norclozapine	49 ± 2.4	40 ± 3.7	44 ± 5.8	51 ± 1.9	49 ± 7.8	50 ± 5.5	60 ± 3.9	60 ± 3.8	60 ± 3.7
Fluoxetine	45 ± 10	28 ± 8.1	36 ± 12.5	44 ± 5.8	30 ± 13.1	37 ± 12.3	63 ± 17.1	48 ± 15.1	56 ± 17.3
Norfluoxetine	27 ± 3.7	25 ± 6.1	26 ± 4.9	32 ± 2	31 ± 9.1	31 ± 6.3	42 ± 8.3	43 ± 5.7	42 ± 6.8
Olanzapine	89 ± 7.7	90 ± 11.8	89 ± 9.5	99 ± 4.1	89 ± 6.4	94 ± 7.0	88 ± 5.6	97 ± 7.4	92 ± 7.9
Quetiapine	69 ± 3.0	59 ± 7.4	64 ± 7.7	74 ± 3.0	65 ± 4.4	70 ± 5.9	77 ± 6.5	73 ± 4.3	75 ± 5.6
N-Desalkylquetiapine	51 ± 2.8	52 ± 5.0	52 ± 3.9	55 ± 1.7	61 ± 6.6	58 ± 5.3	63 ± 5.9	67 ± 6.0	65 ± 6.0
O-Desalkylquetiapine	64 ± 6.6	56 ± 7.9	60 ± 8.1	65 ± 4.5	67 ± 4.5	66 ± 4.3	76 ± 14.2	82 ± 10.3	79 ± 12.3
7-Hydroxyquetiapine	75 ± 8.6	60 ± 16	68 ± 14.4	83 ± 8.8	76 ± 18	79 ± 14	83 ± 10.4	75 ± 25.9	79 ± 19.2
Risperidone	72 ± 3.2	64 ± 5	68 ± 5.9	79 ± 1.9	72 ± 9.3	75 ± 7.3	81 ± 5.8	77 ± 5.0	79 ± 5.7
9-Hydroxyrisperidone	81 ± 5.5	77 ± 9.8	79 ± 7.8	84 ± 3.3	86 ± 6.0	85 ± 4.8	85 ± 7.0	95 ± 6.6	90 ± 8.3
Sulpiride	62 ± 13	70 ± 8.1	66 ± 11.2	77 ± 8.2	80 ± 11.5	79 ± 9.6	74 ± 8.2	64 ± 7.4	69 ± 9.1

Table 3.17: Analyte correction factor for Oral-Eze collection system clinical samples.

Analyte	Result correction factor
Amisulpride	0.83
Aripiprazole	0.40
Dehydroaripiprazole	0.38
Clozapine	0.69
Norclozapine	0.60
Fluoxetine	0.56
Norfluoxetine	0.42
Olanzapine	0.92
Quetiapine	0.75
N-Desalkylquetiapine	0.65
O-Desalkylquetiapine	0.79
7-Hydroxyquetiapine	0.79
Risperidone	0.79
9-Hydroxyrisperidone	0.90
Sulpiride	0.69

3.6 Analyte stability

3.6.1 Initial analyte stability investigation: GBO system

Analytes in the GBO collection fluid were stable at -18 to -20 ºC and at 8 to 10 ºC for 8 weeks, but at ambient temperature (20 ºC; Table 3.18) olanzapine declined at 2 weeks, and 9-hydroxyrisperidone at 5 weeks, with norclozapine possibly starting to decline at 8 weeks. In the course of the clinical trial, the maximum time that the samples were to be stored refrigerated was overnight, and storage at room temperature will be only for hours, therefore the analytes are stable within the required times under the appropriate conditions. Longer-term stability studies for samples stored at -18 to -20 ºC were then undertaken to include the Oral-Eze buffer and other pertinent matrices.

3.6.2 Stability in the GBO and Oral-Eze systems and plasma

Samples were prepared at concentrations (Table 3.3) matching either STD 4 (for 0.1 mol/L hydrochloric acid, deionised water and plasma) or IQC B (for GBO and Oral-Eze buffers) and analysed at the time of preparation. Following storage at -18 to -20 ºC for 6 months, the samples were reanalysed and compared to the nominal concentration (Table 3.19).

Table 3.18: Analyte instability when stored in the GBO buffer at ambient temperature compared to a methanolic equivalent (%; N = 3)

Analyte	1 day	2 days	3 days	1 week	2 weeks	3 weeks	5 weeks	8 weeks
Amisulpride	0	-3	-1	-1	-3	-1	-2	-4
Aripiprazole	2	-1	2	4	-2	-3	5	-8
Dehydro-aripiprazole	3	-2	2	1	-2	-4	-2	-13
Clozapine	-1	-7	1	-4	-9	-8	-12	-6
Norclozapine	-6	-10	1	-5	-11	-4	-15	***-16***
Fluoxetine	2	-5	0	6	-1	-1	-6	8
Olanzapine	0	-2	-1	-9	***-45***	***>100***	***>100***	***>100***
Quetiapine	0	-4	-1	-2	-8	-4	-5	0
N-desalkyl-quetiapine	-4	-7	2	-2	-10	-3	-5	-10
7-Hydroxy-quetiapine	-3	2	-8	-8	-3	-6	-7	-9
O-Desalkyl-quetiapine	-5	-4	1	-4	-8	-7	-11	-3
Risperidone	-2	-3	1	-3	-10	-3	-6	-6
9-Hydroxy-risperidone	1	-1	1	-1	-13	-13	***-28***	***-55***
Sulpiride	-3	-2	-10	-2	2	4	11	3

Table 3.19: Analyte instability in different matrices when stored at -18 to -20 ºC for 6 months. Results outside of acceptable variation (>15 %) are highlighted in bold. N = 4

Analyte	0.1 mol/L hydrochloric acid	GBO buffer	Deionised water	Oral-Eze buffer	Plasma
Amisulpride	-8	6	-6	0	-1
Aripiprazole	9	0	-7	4	-6
Dehydroaripiprazole	11	***56***	-11	-2	4
Clozapine	2	-6	-12	-2	-6
Norclozapine	-6	13	-7	6	-7
Fluoxetine	8	1	-5	10	***34***
Olanzapine	-4	14	-4	-2	-8
Quetiapine	-5	9	0	-9	1
N-Desalkylquetiapine	-14	4	-4	-3	-14
O-Desalkylquetiapine	-4	-4	-3	-4	3
7-Hydroxyquetiapine	-10	11	0	8	-1
Risperidone	-6	2	5	-7	-5
9-Hydroxyrisperidone	-6	-10	-2	-3	-14
Sulpiride	-9	-1	-5	10	-7

The analytes were stable over the time period studied; the only results that were outside of 15 % variation from nominal were those analytes for which there was significant imprecision in the assay, and these results showed an increase, not decrease, in measured analyte concentration.

3.7 Discussion

There have been few papers that study the application of oral fluid in the TDM of antipsychotics (Dumortier *et al*., 1998; Langel *et al*., 2014; Patteet *et al*., 2016; Saracino *et al*., 2010; Fisher *et al*., 2013B). Broadly, these studies have been undertaken using LC-MS/MS as a modification of existing plasma methodologies, in order to attain sensible limits of detection and good analyte selectivity. This process was followed with this work. Two published plasma analytical methods were combined (Fisher *et al*., 2013B; Fisher *et al*., 2012B), clozapine and norclozapine were included, and fluoxetine and norfluoxetine were added. The method developed allowed extraction of all relevant analytes and detection and quantification within a single procedure. The sample preparation technique was then optimised to enable extraction from the buffered oral fluid samples and also work to concentrate the analytes to attain the required sensitivity. The methods take into account the dilution of the analyte in the Oral-Eze buffer, and compensates for the analyte adsorption onto the pad. For the GBO method, a separate spectrophotometric assay was developed to quantify tartrazine within the collection solution, and will be used to calculate the concentration of analyte in the oral fluid.

The oral fluid methods developed were assessed as to whether they were fit-for-purpose. Aripiprazole and dehydroaripiprazole displayed significant inaccuracy and imprecision in both methods, possibly related to the poor analyte stability and solubility identified previously (Fisher *et al*., 2013B) as the variation within-batch was less compared to between different batches. Fluoxetine and norfluoxetine also displayed some imprecision at the lower concentrations in the GBO method, possibly related to this being at the limit of sensitivity for these analytes. In the Oral-Eze method, precision was much poorer for fluoxetine and norfluoxetine, above what would generally be classified as acceptable; in addition the Oral-Eze method showed greater inaccuracy for a number of analytes, especially at the lower concentrations. Thus, accuracy and precision overall were acceptable for the analytes, except for aripiprazole and fluoxetine. Initial plans for the creation of the clinical trial were to obtain samples in collaboration with a local mental health hospital from a variety of patients prescribed the relevant medications, including

children prescribed fluoxetine. However, ethics permission was not attained within the required timeframe, and therefore an alternative source of patient samples was found in an adult inpatient and outpatient facility in South Africa with full ethics approval. This therefore affected the subset of samples collected based on the different selection of prescribed drugs in the patient cohort. As such, the analytes for which the assessment into methodological robustness did not meet the criteria set (aripiprazole and fluoxetine) did not form a large part of the clinical study undertaken to establish the relationship between plasma and oral fluid, since neither fluoxetine nor aripiprazole were prescribed significantly (or at all) in the patient cohort for whom ethics approval was obtained. As such, the methods were fit-for-purpose for the single clinical trial on which it would be used without these analytes meeting the assessment criteria, and no further investigation was undertaken. Process efficiency (overall recovery) was investigated according to the method of Matuszewski *et al*. (2003). Virtually no ion suppression or enhancement was identified for any of the analytes in any matrix. This was likely due to the fact that LLE is a very efficient method of sample preparation with specific analysis by pH-dependent SCX chromatography, and although the extraction recovery varied significantly between analytes and collection device, this was in general well compensated for by the corresponding IS.

Both collection devices investigated relied on the sample being stored in an acidic buffer, and therefore this is likely to help stabilise the basic compounds in question. Short term stability was investigated within the GBO buffer, and showed that all analytes were stable when stored for 8 weeks at 8 to 10 ºC and at -18 to -20 ºC. When stored at ambient temperature (20 ºC), olanzapine was shown to decline after 2 weeks and 9-hydroxyrisperidone at 5 weeks. The stability was therefore acceptable within the required time frame for which samples would be stored for the purposes of the clinical application (overnight refrigerated and maximum of a few hours at room temperature). Samples were prepared in each matrix and stored for a longer time period (6 months) at -18 to -20 ºC to cover the period of the study for sample collection and analysis and represent the longest time the clinical samples would be stored prior to analysis. The results of this study showed that no analytes in any matrix declined by more than 15 %. Those analytes for which variation from the nominal was outside of 15 % displayed an increase in concentration and were analytes for which the normal batch precision was poor, and therefore this variation in response is likely to be analytical variation.

Further investigation could be undertaken to enhance the understanding of between-individual and between-pad variability in the extraction of the analytes from the Oral-Eze pad. For example, a greater number of volunteer analyte-free donors could be tested in order to assess the impact the analyte adsorption onto the pad has on the observed SD (Table 3.16). In addition, a back-calculation experiment could be undertaken to ensure the application of the correction factor is appropriate; this could take the form of applying 1 mL of solution of known concentration in drool oral fluid to the pad in lieu of collecting from the cheek of a patient, but then follow the collection and analysis process as per the protocol. The calculated concentration from this solution can then be used to verify the factor that was applied to patient samples, and quantify the variability in recovery between different pads.

Overall this chapter presents the development and assessment of two oral fluid assays, and the plasma assay. The assays were shown to be fit-for-purpose, when taking into account that aripiprazole and fluoxetine analysis was not integral to the clinical trial being undertaken.

Chapter 4. Potential role of oral fluid in antipsychotic TDM: Clinical study

4.1 Introduction

4.1.1 Oral fluid

The composition of oral fluid, which contains saliva (the largely water-based fluid secreted by the salivary glands) as well as proteins, enzymes, electrolytes and cells, can vary dramatically over the course of a day based on circadian rhythm, degree of hydration, and physiological responses such as stress and oral sensory stimulation (e.g. chewing), and may also be affected by genetics and medication (Aps and Martens, 2005). For a drug to enter saliva it must pass from the bloodstream through the capillary wall by passive or active diffusion, or ultrafiltration. Passive diffusion is the most common route of transfer, hence concentrations of drugs in oral fluid may be affected by the oral fluid pH at the time of collection, and by drug pKa, lipid solubility, molecular weight, unbound concentration in plasma, and salivary binding protein concentration (Aps and Martens, 2005; Drummer, 2008; Patsalos and Berry, 2013). One study in 20 healthy volunteers found unstimulated saliva pH to be 6.8 ± 0.3, but high salivation rates produced more alkaline pH, and low salivation rates more acidic pH with lower buffering capacity (Bardow *et al*., 2000); a similar study found the mean pH across all sites in the mouth to be 6.78 ± 0.04, with the mean pH specifically in the cheek area of 6.28 ± 0.36 (Aframian *et al*., 2006).

Further potential variables in the collection of oral fluid samples include stimulation of saliva production during sample collection, variation in the volume of sample taken, inhomogeneity of the oral fluid collected, and oral cavity contamination with tablet residue. A study to quantify different salivary proteins in oral fluid using a number of commercially available collection devices showed significant differences in both the analyte concentrations and salivary flow rates between the different collection techniques (Topkas *et al*., 2012), in turn showing that each collection device may collect a non-comparative oral fluid sample.

Comparison of commercially available oral fluid collection devices and the recovery and stability of a number of different drugs of abuse showed that there is a large variability in the suitability of the varying collection devices (Crouch *et al*., 2008; Langel *et al*., 2008). The authors of one

study recommend thorough testing prior to utilising any collection device to ensure that the recovery and stability of the analytes in question meet requirements, where limitations can include contamination of the instrumentation from additives in the collection device such as the buffer; they concluded that devices which rely on a buffer overall had improved recovery and stability, and the GBO device performed well in all areas of testing (Langel *et al*., 2008). Moreover, use of a collection solution could also facilitate oral fluid sampling in patients that produce small quantities of saliva. Dilution of the (normally viscous) oral fluid in the collection solution could help accurate pipetting. On the other hand, dilution of the oral fluid collected may limit sensitivity.

Oral fluid has been used for TDM of some weakly acidic/neutral drugs such as anticonvulsants (Patsalos and Berry, 2013), as well as regularly in roadside devices for drugs of abuse testing (Crouch *et al*., 2004; Crouch *et al*., 2008; Langel *et al*., 2008; Langman *et al*., 2007; Willie *et al*., 2009). However, for protonatable (basic) drugs, it is likely that changes in oral fluid pH during sample collection affect the presumed plasma:oral fluid equilibrium (Horning *et al*., 1977; Kato *et al*. 1993).

4.1.2 SGAs in oral fluid

Many of the SGAs are basic drugs (pKa ~8-9; Table 1.1), therefore changes in oral fluid pH may impact the diffusion of these analytes across the salivary glands and into oral fluid. In addition, many of the SGAs act on the adrenergic and cholinergic systems, which may in turn affect the composition of oral fluid compared to healthy individuals (Taylor *et al.*, 2015).

In oral fluid collected via the drool method (unstimulated oral fluid) in 90 patients prescribed a variety of SGAs, there was a relatively poor correlation (R^2 = 0.3–0.7) between the plasma and oral fluid concentrations measured (Fisher *et al*., 2013A). Hence, unstimulated oral fluid was unable to predict plasma concentrations (Fisher *et al*., 2013A; Patteet *et al.,* 2015).

In a small study of risperidone concentrations in paediatrics using oral fluid collected by Sarstedt Salivette (which will stimulate salivary flow to some degree) there was a good correlation (R^2 = 0.93) between plasma and oral fluid risperidone concentrations when plotted on a logarithmic scale (Aman *et al*., 2007). However, other studies have shown poorer relationships, and wide between-patient variability between plasma and oral fluid for both risperidone and 9-hydroxyrisperidone concentrations as well as some other antipsychotic drugs

(Flarakos *et al*., 2004; Saracino *et al*., 2010; Mandrioli *et al*., 2011; Patteet *et al.,* 2016). The wide variability reported in these studies is analogous to results from work on other basic analytes, including antidepressants and some drugs of abuse (de Castro *et al*., 2008; Wille *et al*., 2009).

Whilst the existing limited available information overall show a relatively poor correlation between plasma and oral fluid concentrations of the analytes of interest, there may be some potential for applying oral fluid measurement if the between-patient and between-collection variation in the plasma:oral fluid ratio can be minimised. One possible explanation is variation in salivary pH during sample collection affecting these protonatable basic analytes. Use of a buffered oral fluid collection system may reduce the variability in oral fluid pH and thus improve the relationship between oral fluid and plasma analyte concentrations of basic analytes both between collections and between individuals, as well as potentially improving analyte stability (Langel *et al*., 2008).

4.1.3 GBO oral fluid collection device

The GBO collection device relies on holding a collection buffer in the mouth that will buffer the oral cavity to a defined pH during collection (approx. pH 4-6; Greiner Bio One, 2011). This means that theoretically all samples are collected and maintained under the same conditions irrespective of between-collection and between-individual variation in salivary pH and flow-rate. The amount of oral fluid can be accurately quantified by measuring the degree of dilution of tartrazine dye contained within the collection buffer, as discussed in Chapter 3. Correcting the analyte result quantified in the collection fluid by the amount of oral fluid contained within the collection fluid enables accurate determination of the concentration of analyte present in oral fluid.

Limitations with this collection device including potential contamination of the sample from drug adsorbed onto the teeth are the same as with all oral fluid collections. However, in addition this device is unsuitable for use in patients with intolerance to aspirin or benzoic acid and in asthmatic patients due to the presence of, and therefore potential allergic response to, tartrazine (Greiner Bio One, 2011). Analyte stability has also been identified as an issue for the drugs of interest in unmodified oral fluid samples (Fisher *et al*., 2013C), therefore the impact of the sample being stored within a buffered solution prior to analysis had to be investigated (Chapter 3).

This collection device has been used in a wide variety of published applications including early detection of herpes simplex virus (Lackner *et al*., 2010), extraction of salivary DNA for the diagnosis of genetic disorders (Paar *et al*., 2014), investigation of salivary biomarkers chromogranin A and amylase in periodontitis (Haririan *et al*., 2012), quantification of codeine (Coucke *et al*., 2015), and investigation into detection of metamfetamine enantiomers following administration of a decongestant nasal inhaler (Newmeyer *et al*., 2015). However, there has been no previous detailed study in its use for the TDM of basic drugs.

4.1.4 Oral-Eze oral fluid collection device

The Oral-Eze device consists of a collection pad that is held in the mouth against the cheek until the indicator on the handle turns blue to indicate that 1 mL oral fluid has been collected (or at a maximum of 10 minutes). The pad is then detached into a tube which contains 2 mL of a buffer (approx. pH 6) for storage until analysis (Thermo Fisher Scientific, 2013; Chapter 3).

This collection device does not collect oral fluid under defined pH conditions; however, it theoretically collects a defined oral fluid volume in a stimulated method from a fixed part of the mouth (cheek), so this may be able to counteract the variation in salivary pH. The presence of the buffer post-collection may improve analyte stability on storage. Limitations include the possible variability of 1 mL collection volume, and the need to consider analyte adsorption to the pad affecting the equilibrium between the collected oral fluid on the pad and the buffered solution that is analysed.

Published applications of this collection device include quantifying cocaine and benzoylecgonine following intravenous cocaine administration (Ellefson *et al*., 2016) and investigation into the concentrations and stability of cannabis and metabolites in oral fluid samples (Anizan *et al*., 2015; Desroisiers *et al*., 2014; Samano *et al*., 2015).

4.1.5 Aims of the chapter

Little work has been undertaken to apply oral fluid collection devices to TDM of antipsychotic drugs. Theoretically use of a buffered collection device should minimise variations in oral fluid pH during sample collection, and hence may minimise the impact of this variable on the use of oral fluid for the TDM of basic drugs.

A clinical trial was set up in conjunction with Stellenbosch University following full ethical approval (Appendix A) to obtain oral fluid and plasma samples from patients prescribed clozapine along with other relevant drugs.

This chapter will present the results from paired sets of plasma and oral fluid samples collected using both oral fluid collection devices. Data analysis will be undertaken to investigate whether use of the GBO system minimises between-patient and between-collection variability in the relationship between plasma and oral fluid analyte concentrations, as compared to post-collection storage in buffer.

4.2 Materials and methods

4.2.1 Ethics

Ethics was applied for and granted by the Health Research Ethics Committee for Stellenbosch University, South Africa (reference S13/11/227), for the collection of oral fluid and plasma in patients prescribed clozapine (Appendix A).

Patient consent was required prior to collection of samples (Appendix A), and the patients' doctor was required to agree that the patient was competent to give consent.

Patients were eligible if they were over 18, did not suffer from aspirin or benzoic acid allergy or have a history of asthma, and if they were prescribed the tablet formulation of clozapine; patients taking the suspension formulation were not eligible due to the risk of drug adsorption onto teeth. Repeat samples from the same patient were permitted provided there was at least one day between collections.

Full protocols used during the preparation and collection of samples are given in Appendix A. Collection of oral fluid samples was undertaken according to the manufacturer's guidelines (Greiner Bio One, 2011; Thermo Fisher Scientific, 2013).

4.2.2 Sample collection

Patients were not permitted to eat or drink for 30 and 10 min, respectively, prior to oral fluid collection. The first sample collected was using the Oral-Eze device held against the side of the cheek until the indicator turned blue, or at 10 min. The pad was dispensed in the collection buffer and mixed. Following this, the GBO sample was collected, where the patient rinsed their mouth with the wash solution, then held the collection buffer in the mouth for 2 min prior to

being spat into the beaker and collected into the collection tube which was stored at -18 ºC to -20 ºC until analysis. Finally, blood was taken into a BD dipotassium ETDA tube (at least 3 mL). Plasma was separated off (1500 *g*, 5 mins) and stored in polypropylene tubes at -18 ºC to -20 ºC until analysis. After overnight storage, the pad was plunged from the Oral-Eze buffer, and the buffer collected into 2 mL tubes and stored at -18 ºC to -20 ºC with the other 2 samples. All three sets of samples were labelled with the trial sample ID and stored together in a labelled bag. At the end of patient sample collection, all sample sets were sent together by courier on dry ice to the Toxicology Unit where they were stored at -18ºC to -20 ºC until analysed. Patient age and sex, time sample taken post-dose, and drug dosing schedules were collated for clozapine and other relevant co-prescribed medications, and stored anonymised under the trial patient and sample ID.

4.2.3 Analytical methods

The analytical methods used were those as described in Chapter 3. Antipsychotic concentrations calculated from the LC-MS/MS method described had to be corrected for the recovery from the pad for the Oral-Eze (as per section 3.5.8) and for the GBO device corrected for the volume of oral fluid within the collection fluid as calculated by the tartrazine concentration (as per section 3.3.3). Assay performance matched during the assessment, and all IQCs were within accepted limits for analytes that were measured in patient samples.

4.2.4 Statistical methods

Statistical analysis was undertaken using Excel Analyse-It (v2.30 Excel 12+) and SPSS (Statistics v23), according to the protocols set out in Chapter 2.2.2.

4.3 Patient demographics

Overall, 200 sets of samples (only 198 GBO samples) were collected from 112 participants; of these participants 31 provided a single set, 74 provided two sets and 7 provided three sets. Full patient demographics are given in Table 4.1.

The other relevant medication dosage regimes were also recorded, and the results for these samples collated. For each sample set, all three sample types were collected (plasma, GBO and Oral-Eze), except in two cases where the GBO system was not collected.

4.4 GBO oral fluid content

A total of 198 GBO samples were received; the median oral fluid content was 52 % (range 13-86 %), with the 5th, 25th, 75th and 95th percentiles as 22, 37, 63 and 80 %, respectively. The oral fluid content of the collection fluid was not normally distributed (Shapiro Wilk 0.981, $p<0.01$), although the plot shows a good distribution especially in the central results, and there was a slight non-significant trend towards a lower oral fluid content with increasing age (Figure 4-1).

The influence of independent variables (age, sex and clozapine dose) on oral fluid content was investigated by regression analysis. Model strength was very low; hence, oral fluid content in the GBO collection fluid could not be predicted by age, sex or clozapine dose. Within-patient variability in oral fluid content was investigated in three separate samples from 7 patients. The results of these samples (Table 4.2) show some variability between collections, even from the same patient, therefore the presence of the buffer in-mouth does not completely reduce between-collection variability in the oral fluid collected.

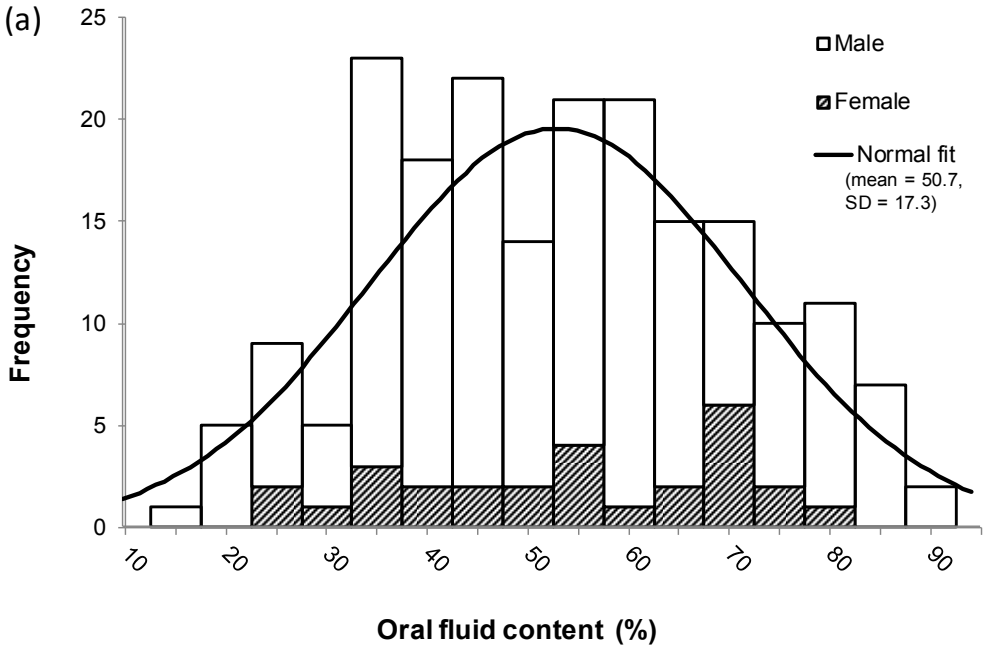
Table 4.1: Patient demographics of collected oral fluid sample sets

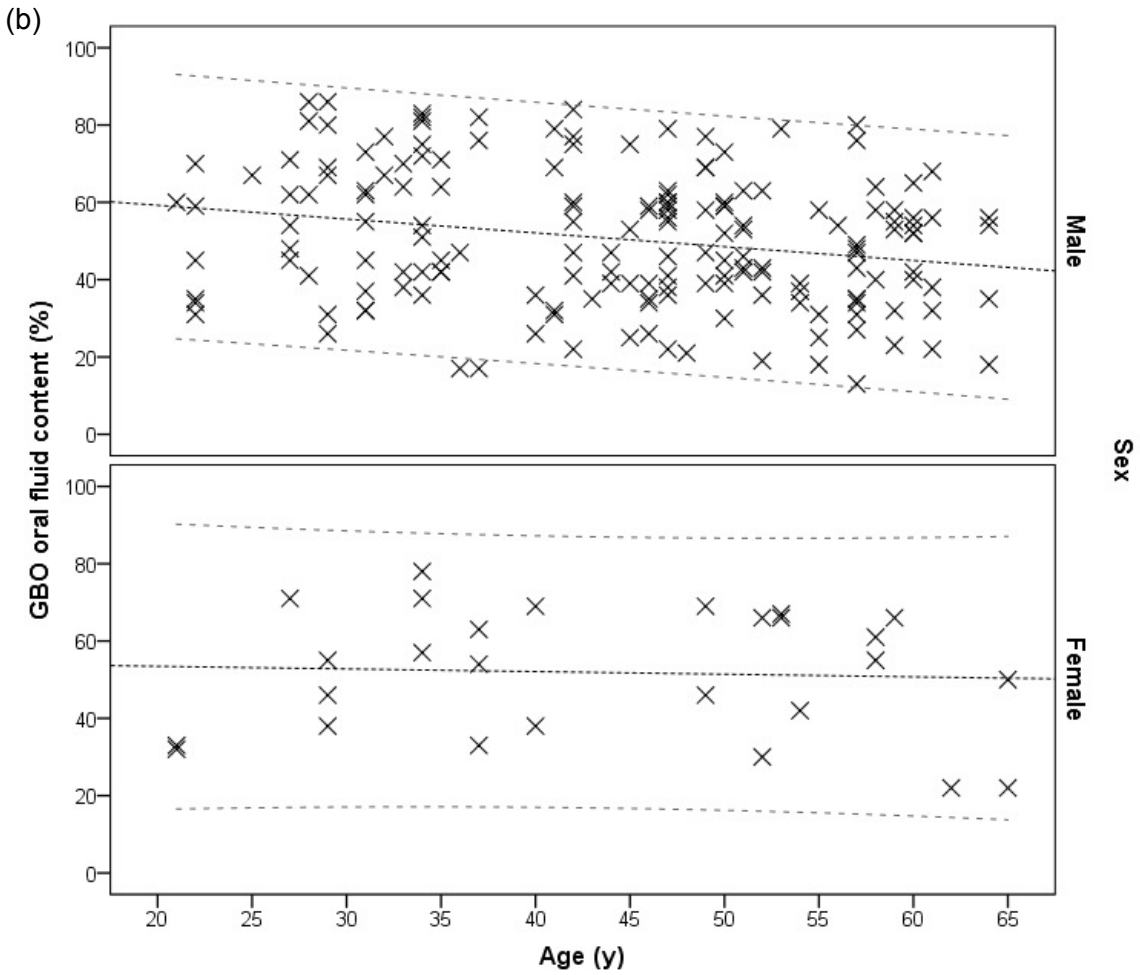
Replicates	**Number**		**Age (median, range)**	
	Male	**Female**	**Male**	**Female**
First	96	16	47 (21 - 65)	44 (21 – 65)
Second	71	10	46 (22 - 64)	44 (21 – 65)
Third	6	1	40 (27 – 49)	34
TOTAL	*173*	*27*	*47 (21 – 65)*	*44 (21 – 65)*

Table 4.2: Consistency observed in oral fluid content from three separate collection sets

Patient: Sex, age	**Time between collections, d**	**Oral fluid content (%)**			**RSD (%)**
		First sample	**Second sample**	**Third sample**	
Male, 47	119, 56	58	56	38	21
Male, 47	5, 170	55	63	60	7
Male, 29	14, 7	67	69	80	9
Male, 27	14, 7	48	54	45	10
Female, 34	14, 7	57	71	78	15
Male, 34	14, 7	75	72	36	35
Male, 49	14, 7	77	69	69	6

Figure 4-1: Oral fluid content in the GBO patient samples
a) Normal fit distribution for GBO oral fluid content in males and females; b) Breakdown of oral fluid content in the GBO collection system by age and sex. Lines show line of fit and 95% confidence intervals





4.5 Clozapine and norclozapine

The median dose was 400 (range 50–800) mg/d. Median sampling time post-dose was 13 (range 11–26) h, with 16 samples not having a specific collection time recorded (minimum 10 h post-dose). Clozapine dosage was once-daily (N = 78); twice-daily (N = 114); and thrice-daily (N = 8).

Clozapine and norclozapine were not detected in plasma and in both oral fluid samples from a male outpatient aged 28 yr (prescribed clozapine dose 400 mg/d). Fourteen days previously his plasma clozapine and norclozapine were 0.34 and 0.13 mg/L, respectively. His relapse having become apparent, hospital admission and re-titration were arranged promptly.

4.5.1 Correlation between sample types

The full results are summarised in Table 4.3. Correlations between plasma and both oral fluid results were similar between clozapine and norclozapine for GBO and Oral-Eze compared to plasma (0.74 - 0.79), whereas those between both oral fluid collection devices were slightly higher for clozapine and norclozapine (0.84 and 0.85, respectively; Table 4.4; Figure 4-2; Figure 4-3; and Figure 4-4).

Table 4.3: Clozapine and norclozapine concentrations in plasma and in oral fluid.
(a) summary data; (b) between sample-type ratios

(a)	Clozapine (mg/L)			Norclozapine (mg/L)		
	Plasma[1]	GBO[2]	Oral-Eze[1]	Plasma[1]	GBO[2]	Oral-Eze[1]
Minimum	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
25th percentile	0.25	0.17	0.21	0.11	0.08	0.14
Median	0.43	0.32	0.40	0.20	0.15	0.26
75th percentile	0.86	0.62	0.71	0.32	0.27	0.43
Maximum	2.30	1.73	2.72	1.59	0.98	1.19
Mean [3] (SD)	0.60 (0.46)	0.44 (0.36)	0.53 (0.45)	0.26 (0.21)	0.19 (0.16)	0.32 (0.24)

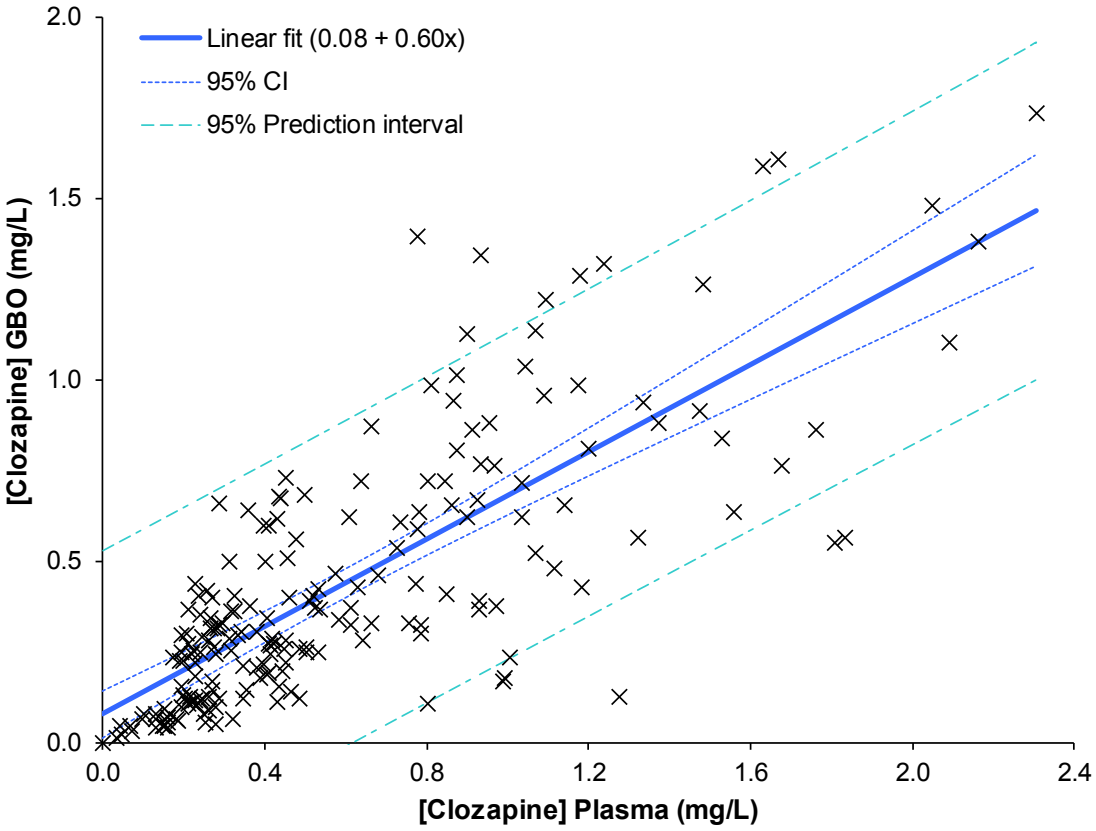
(b)	Clozapine			Norclozapine		
	Plasma: GBO [2]	Plasma: Oral-Eze [1]	GBO: Oral-Eze [2]	Plasma: GBO [2]	Plasma: Oral-Eze [1]	GBO: Oral-Eze [2]
Minimum	0.44	0.33	0.15	0.38	0.23	0.15
25th percentile	0.94	0.85	0.63	0.94	0.57	0.46
Median	1.47	1.19	0.79	1.35	0.79	0.57
75th percentile	2.11	1.69	1.11	2.15	1.09	0.74
Maximum	10.15	9.13	4.08	6.76	4.98	2.19
Mean[3] (SD)	1.75 (1.21)	1.39 (0.97)	0.91 (0.48)	1.64 (1.01)	0.92 (0.56)	0.63 (0.30)

[1] N = 200; [2] N = 198; [3] = one set of samples clozapine and norclozapine not detected and removed from data

Table 4.4: Correlations between plasma and oral fluid concentrations.
* p<0.01

	Spearman correlation		
	Plasma:GBO	Plasma:Oral-Eze	GBO:Oral-Eze
Clozapine	0.78 *	0.79 *	0.84 *
Norclozapine	0.74 *	0.79 *	0.85 *

Figure 4-2: Plot of oral fluid and plasma concentration correlations for clozapine and norclozapine. Trendlines, 95 % confidence and prediction intervals shown.



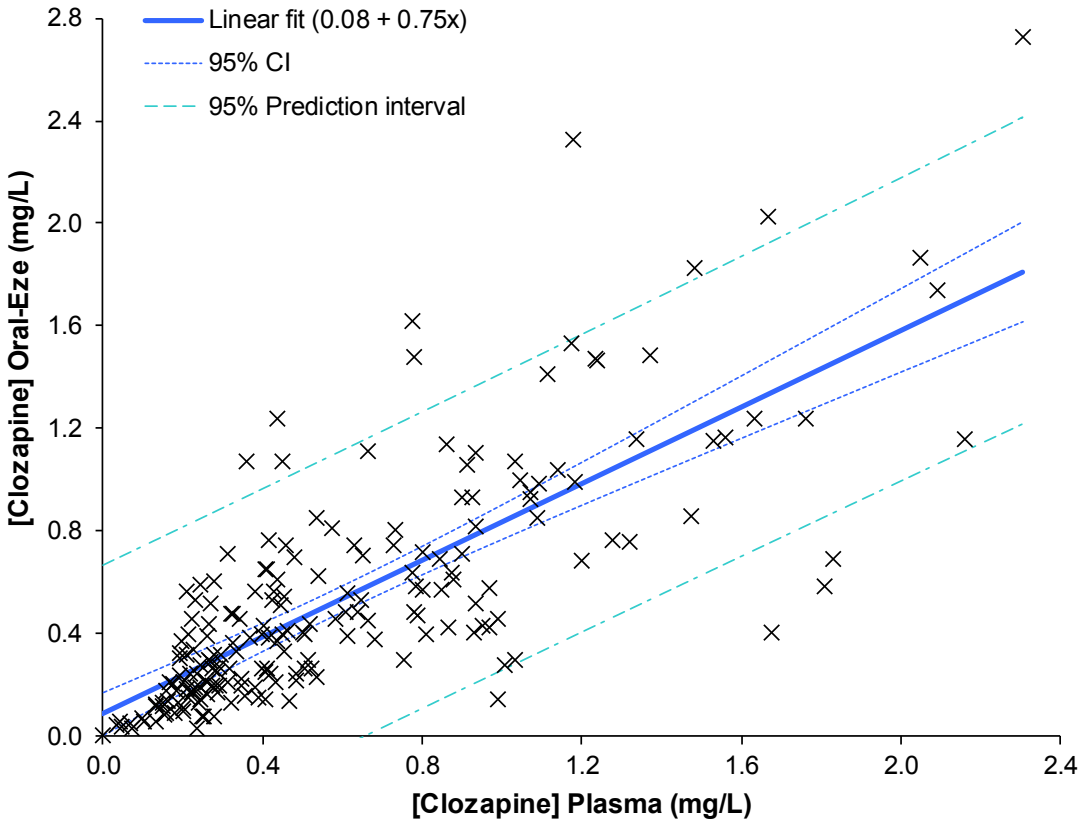
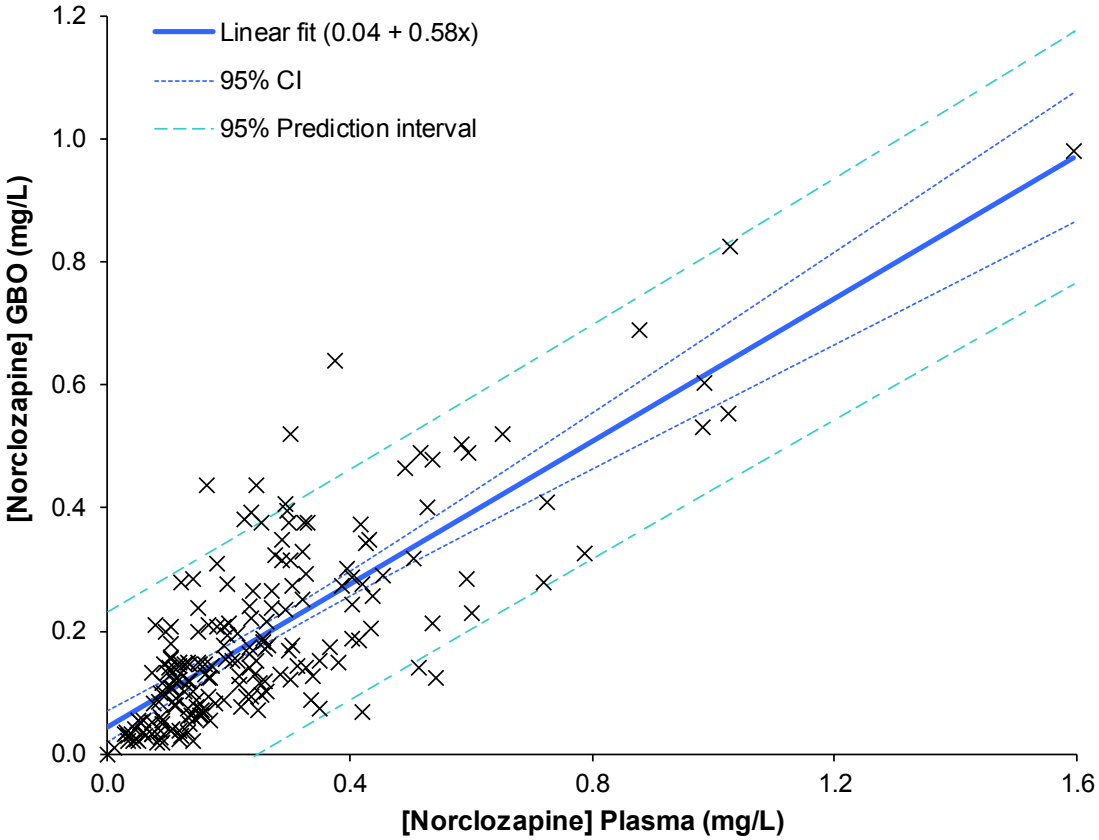


Figure 4-2 (cont.)



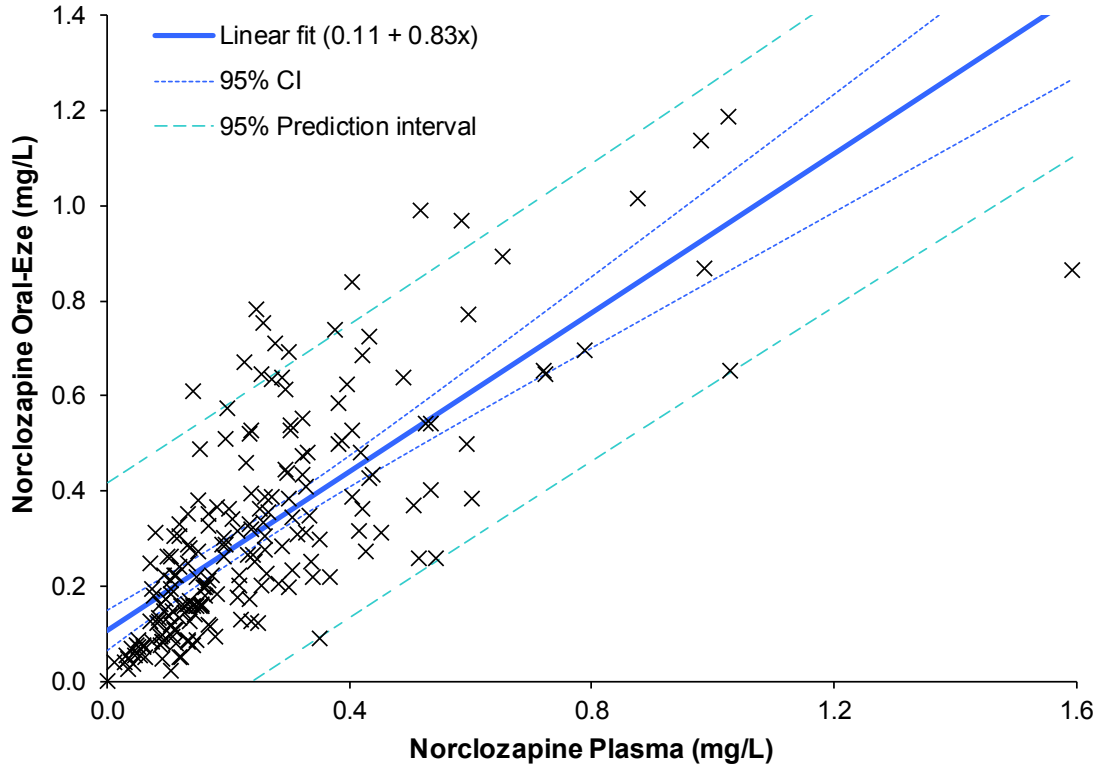
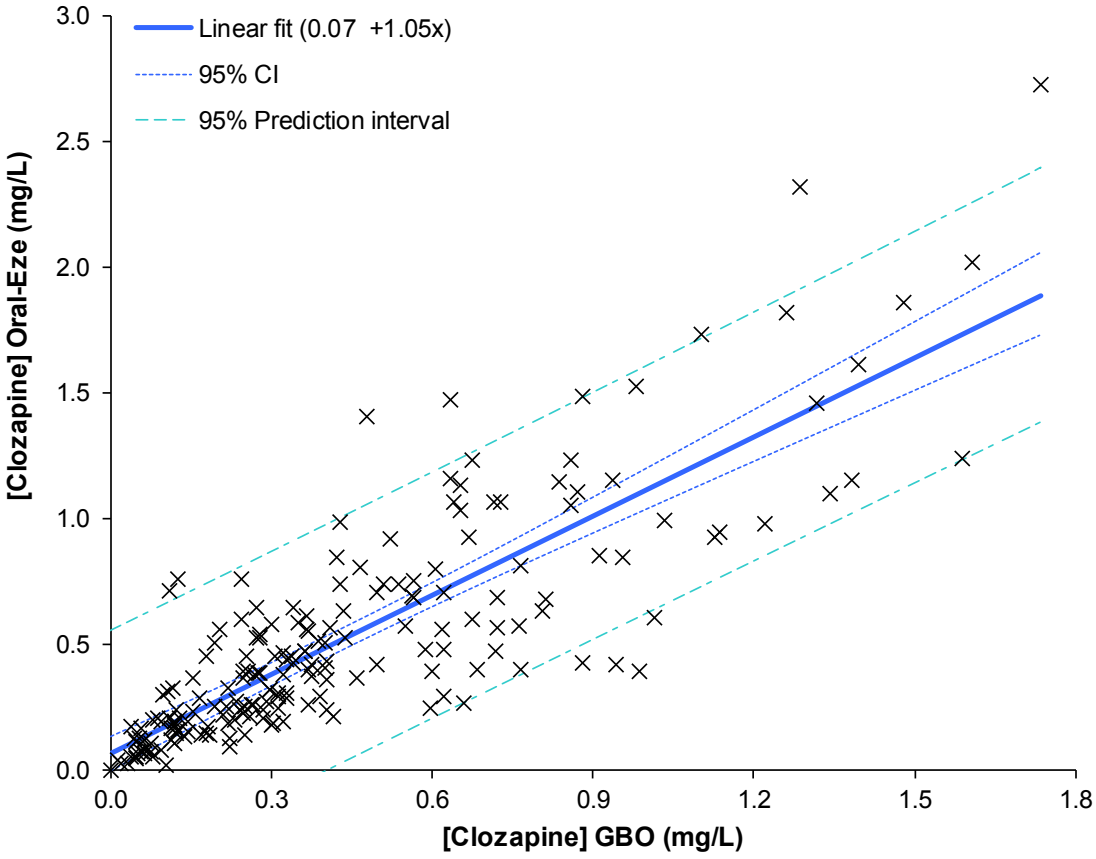


Figure 4-3: Plot of oral fluid concentration correlations for clozapine and norclozapine. Trendlines, 95 % confidence and prediction intervals shown.



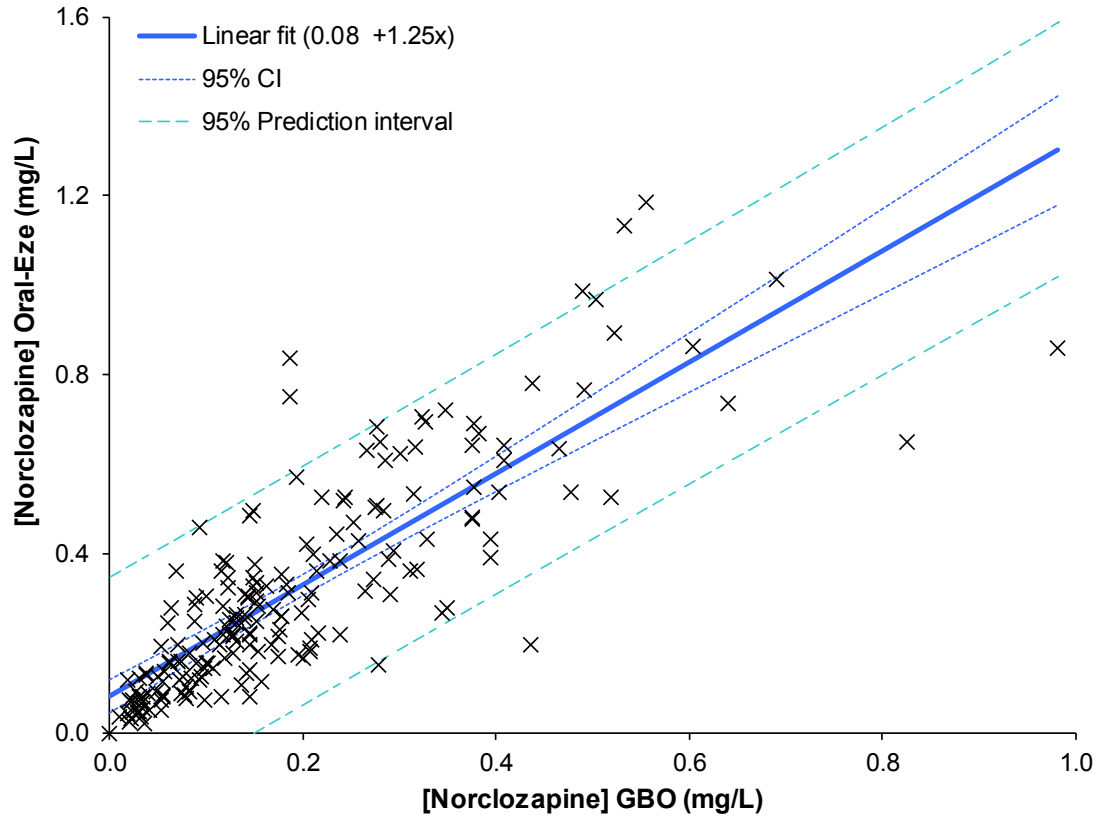
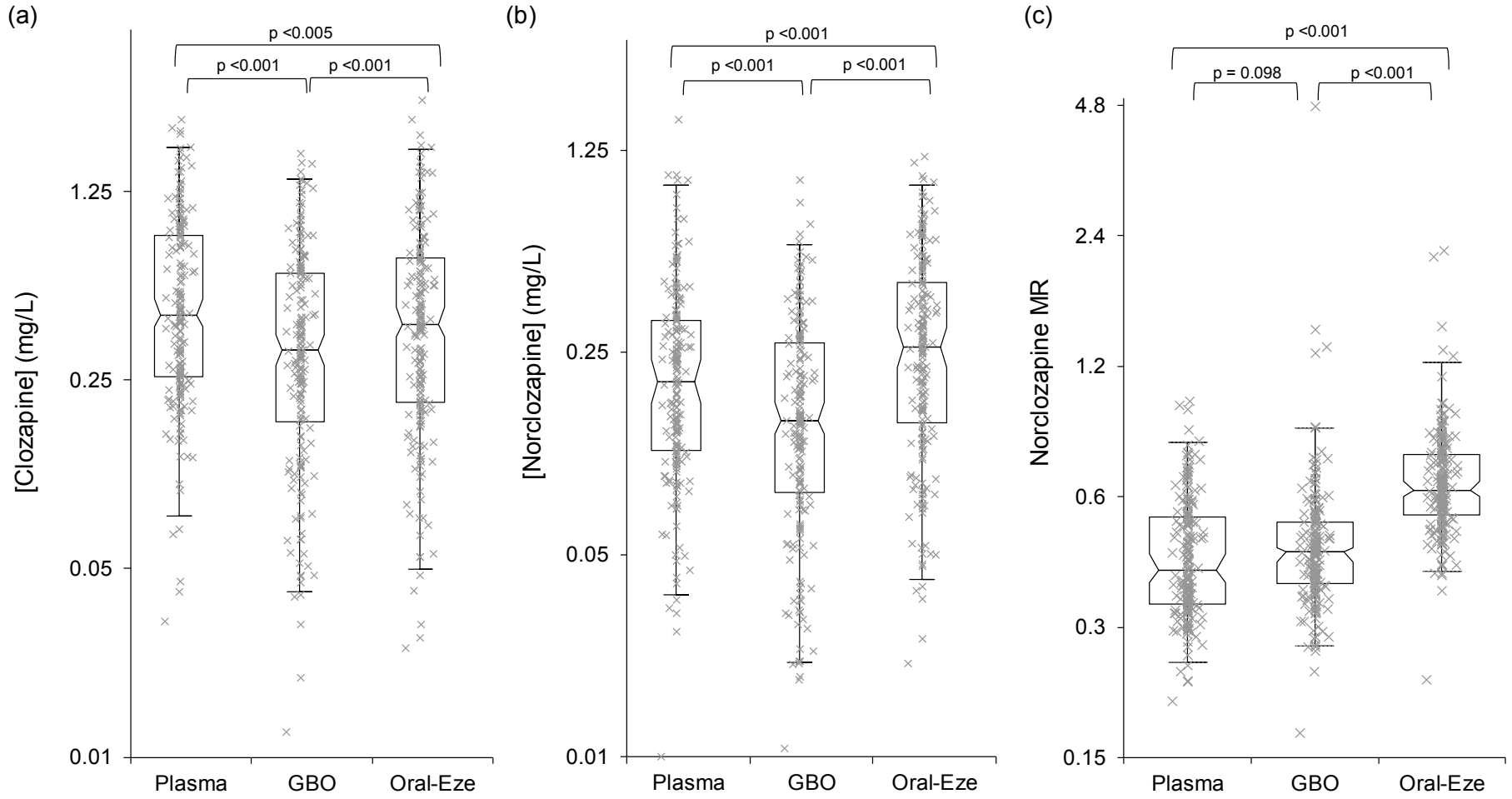


Figure 4-4: Summary patient clozapine results comparison between the difference matrices.
(a) clozapine, (b) norclozapine, (c) norclozapine MR ratio. Box = median and inter-quartile range, whiskers = 95% CI; paired t-test p-value given between each dataset for each analyte. Norclozapine MR calculated as norclozapine plasma concentration divided by clozapine plasma concentration. Plotted on log scale.



There was a significant difference between males and females (Figure 4-5) in the ratio of plasma:Oral-Eze concentrations for both clozapine and norclozapine, and in the plasma clozapine concentration. However, plasma norclozapine concentrations and all variables including dose ($p = 0.76$) and oral fluid content ($p = 0.68$) were not significantly different between males and females.

4.5.2 Regression analysis

Regression analyses were undertaken for both clozapine (Table 4.5) and norclozapine (Table 4.6) to investigate the influence on oral fluid concentrations of independent variables age, sex, clozapine dose, time since last dose, the GBO oral fluid content. GBO oral fluid content was included for the regression the Oral-Eze studies as well as the GBO samples in case this variable could be used as a surrogate marker for salivary flow rate. The difference in plasma compared to oral fluid clozapine and norclozapine concentrations was also investigated, to see if there was a proportional impact of the plasma concentration on the oral fluid result.

Plasma analyte concentration was the most relevant variable in predicting the oral fluid concentration for both devices, explaining between 60 % and 70 % of the variation, although age and clozapine dose were also significant. For clozapine, for an increase in plasma concentration of 1 mg/L, there was a predicted increase in 0.53 and 0.66 mg/L for GBO and Oral-Eze concentrations, respectively. For norclozapine, an increase in plasma concentration of 1 mg/L predicted an increase of 0.49 and 0.65 mg/L for GBO and Oral-Eze, respectively. The model strength for these regression analyses were low as would be expected since there was wide variability in the results (Figure 4-2).

The difference in plasma:oral fluid concentration for clozapine and norclozapine was not reliably modelled since the result predicted by the equation only displayed 15-21 % correlation to the observed result, as expected from the poor correlation between the plasma:oral fluid concentration difference and age, dose and plasma analyte concentration (Figure 4-6, Figure 4-7 and Figure 4-8, respectively).

Figure 4-5: Clozapine and norclozapine concentrations between males and females, and the ratio in plasma:Oral-Eze concentrations.
Box = median and inter-quartile range, whiskers = 95% CI; paired t-test p-value given between each dataset for each analyte. Plotted on log scale.

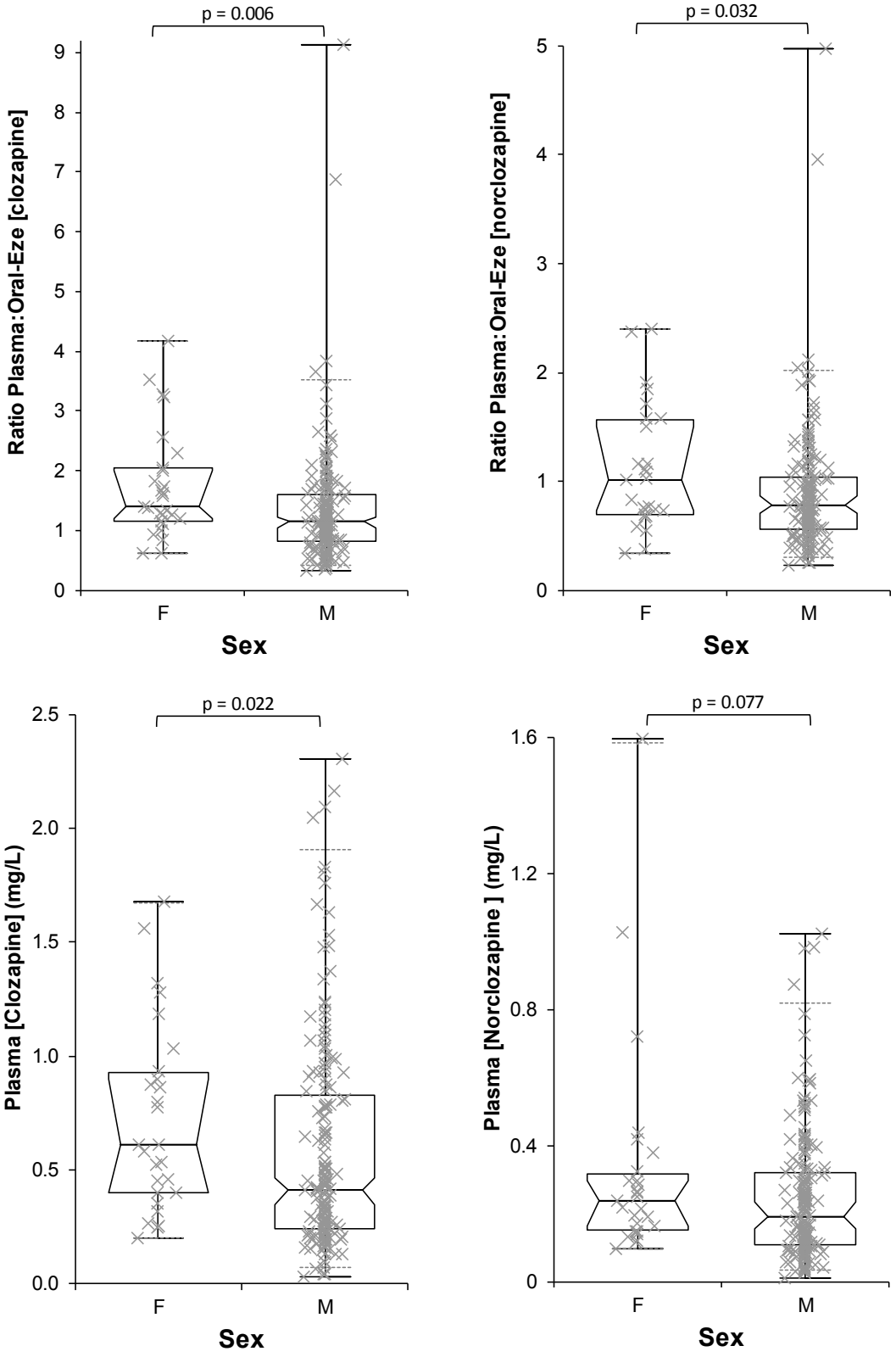


Table 4.5: Regression data for clozapine concentration prediction

Dependent variable	Model * (R, R^2)	Significant independent variables	Predictor *			
			B	95 % CI	Beta	*P* value
GBO concentration	0.80, 0.64	Plasma clozapine concentration (mg/L)	0.53	0.46, 0.61	0.69	<0.001
		Age	0.005	0.002, 0.008	0.16	<0.001
		Clozapine dose (100 mg/d)	0.030	0.008, 0.052	0.14	0.008
		(constant)	-0.20	-0.36, -0.08	-	0.002
	Excluded GBO oral fluid content (p=0.69), time since last dose (p=0.33), sex (p=0.29)					
Oral-Eze concentration	0.79, 0.62	Plasma clozapine concentration (mg/L)	0.66	0.56, 0.76	0.67	<0.001
		Age	0.005	0.002, 0.008	0.13	0.004
		Clozapine dose (100 mg/d)	0.041	0.013, 0.070	0.15	0.004
		(constant)	-0.25	-0.43, -0.06	-	0.008
	Excluded GBO oral fluid content (p=0.80), time since last dose (p=0.72), sex (p=0.02)					
Difference plasma:GBO	0.42, 0.18	Plasma clozapine concentration (mg/L)	-26.9	-40.4, -13.5	-0.31	<0.001
		Age	1.22	0.77, 1.67	0.35	<0.001
		Clozapine dose (100 mg/d)	5.75	2.01, 9.49	0.23	0.003
		(constant)	-82.7	-106.7, -58.8	-	<0.001
	Excluded sex (p=0.50), GBO oral fluid content (p=0.15), time since last dose (p=0.04)					
Difference plasma: Oral-Eze	0.39, 0.15	Plasma clozapine concentration (mg/L)	-36.2	-53.3, -19.1	-0.33	<0.001
		Age	1.24	0.66, 1.81	0.28	<0.001
		Clozapine dose (100 mg/d)	7.88	3.10, 12.66	0.25	0.001
		(constant)	-68.6	-99.252, -37.997	-	<0.001
	Excluded GBO oral fluid content (p=0.76), time since last dose (p=0.12), sex (p=0.04)					

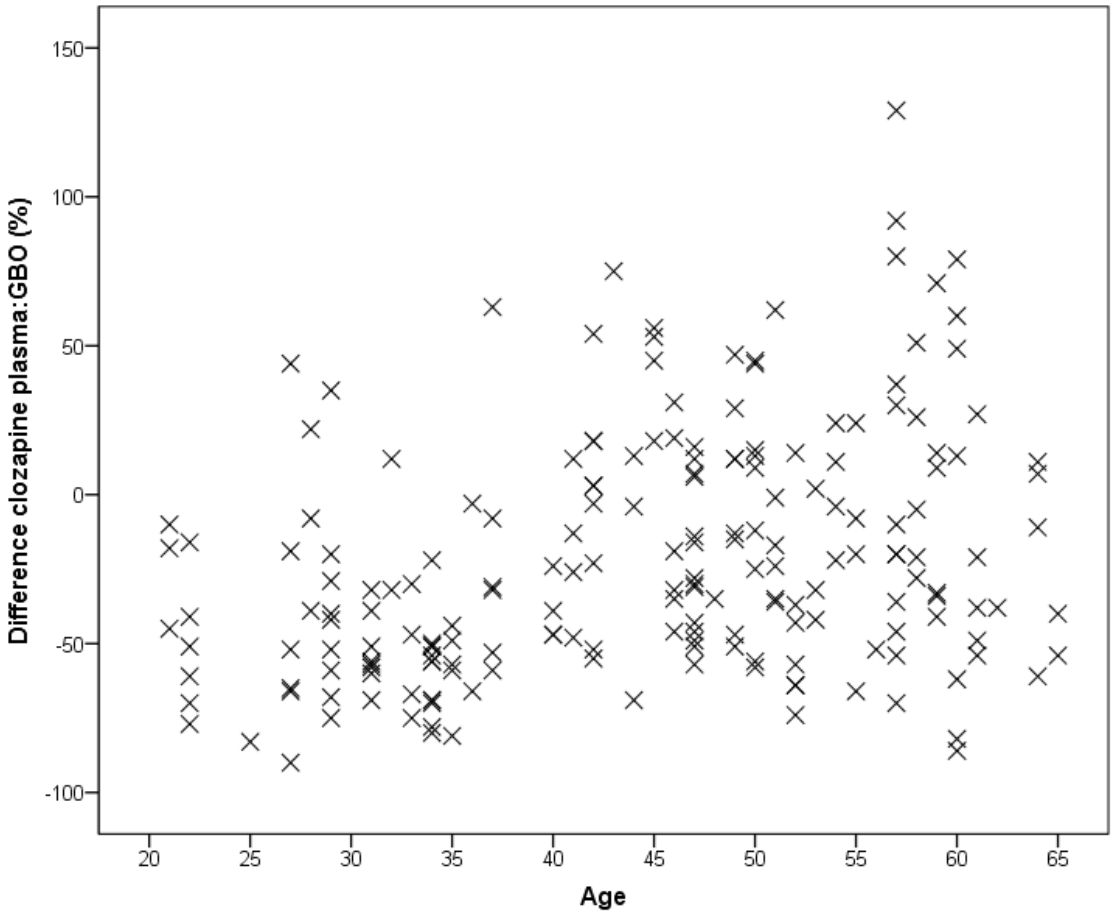
* model and predictors explained in Section 2.2.2.1

Table 4.6: Regression data for norclozapine concentration prediction

Dependent variable	Model * (R, R^2)	Significant independent variables	Predictor *			
			B	95 % CI	Beta	*P* value
GBO concentration	0.83, 0.70	Plasma norclozapine concentration (mg/L)	0.49	0.42, 0.56	0.67	<0.001
		Age	0.003	0.002, 0.004	0.21	<0.001
		Clozapine dose (100 mg/d)	0.017	0.009, 0.026	0.18	<0.001
		(constant)	-0.13	-0.18, -0.07	-	<0.001
	Excluded GBO oral fluid content (p=0.33), sex (p=0.30), time since last dose (p=0.16)					
Oral-Eze concentration	0.80, 0.64	Plasma norclozapine concentration (mg/L)	0.65	0.54, 0.77	0.59	<0.001
		Clozapine dose (100 mg/d)	0.04	0.02, 0.05	0.27	<0.001
		Age	0.003	0.001, 0.005	0.15	0.001
		(constant)	-0.13	-0.22, -0.03	-	0.008
	Excluded GBO oral fluid content (p=0.59), time since last dose (p=0.52), sex (p=0.14)					
Difference plasma:GBO	0.45, 0.21	Plasma norclozapine concentration (mg/L)	-81.2	-113.9, -48.5	-0.38	<0.001
		Age	1.44	0.94, 1.94	0.37	<0.001
		Clozapine dose (100 mg/d)	6.63	2.43, 10.8	0.24	0.002
		(constant)	-86.4	-113.0, -59.8	-	<0.001
	Excluded sex (p=0.40), time since last dose (p=0.21), GBO oral fluid content (p=0.06)					
Difference plasma: Oral-Eze	0.43, 0.19	Plasma norclozapine concentration (mg/L)	-149.6	-201.2, -98.0	-0.45	<0.001
		Age	1.69	0.91, 2.48	0.28	<0.001
		Clozapine dose (100 mg/d)	12.23	5.61, 18.86	0.28	<0.001
		(constant)	-44.3	-86.2, -2.3	-	0.04
	Excluded GBO oral fluid content (p=0.68), time since last dose (p=0.43), sex (p=0.31)					

* model and predictors explained in Section 2.2.2.1

Figure 4-6: Plot of age against difference between plasma and oral fluid clozapine and norclozapine concentrations.



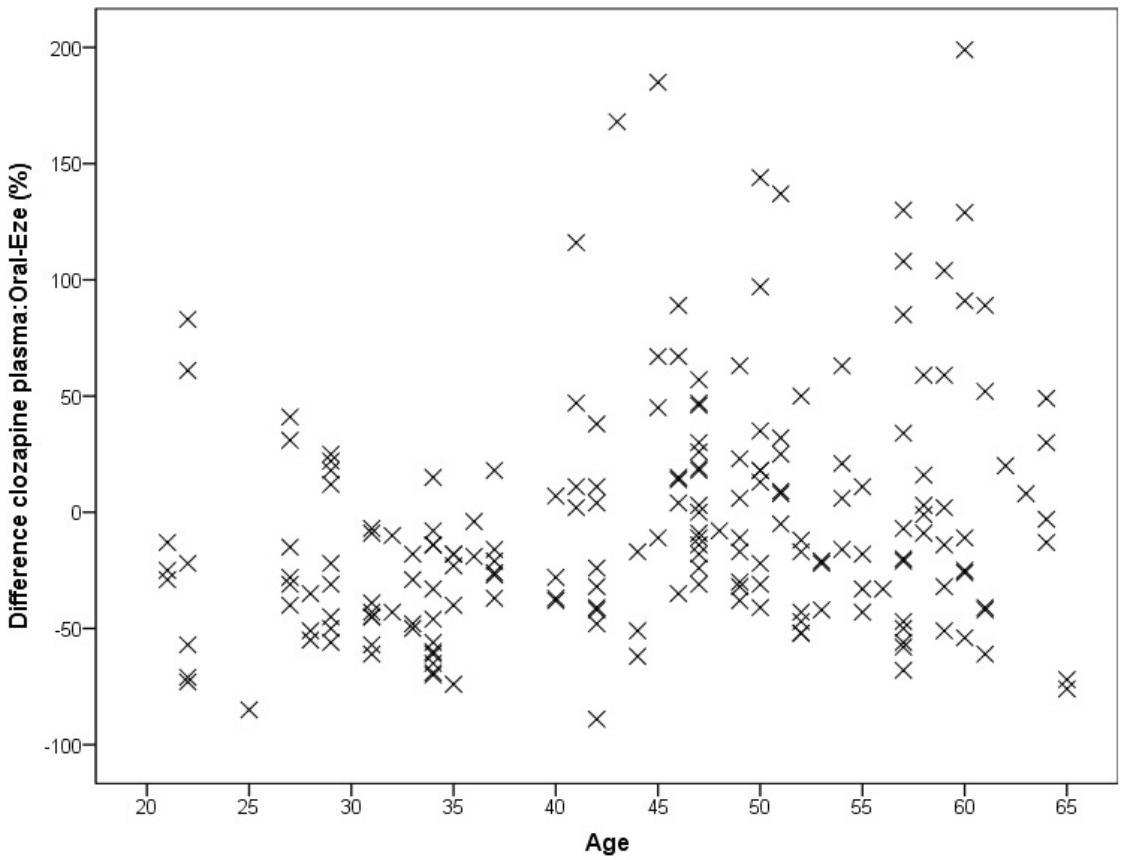
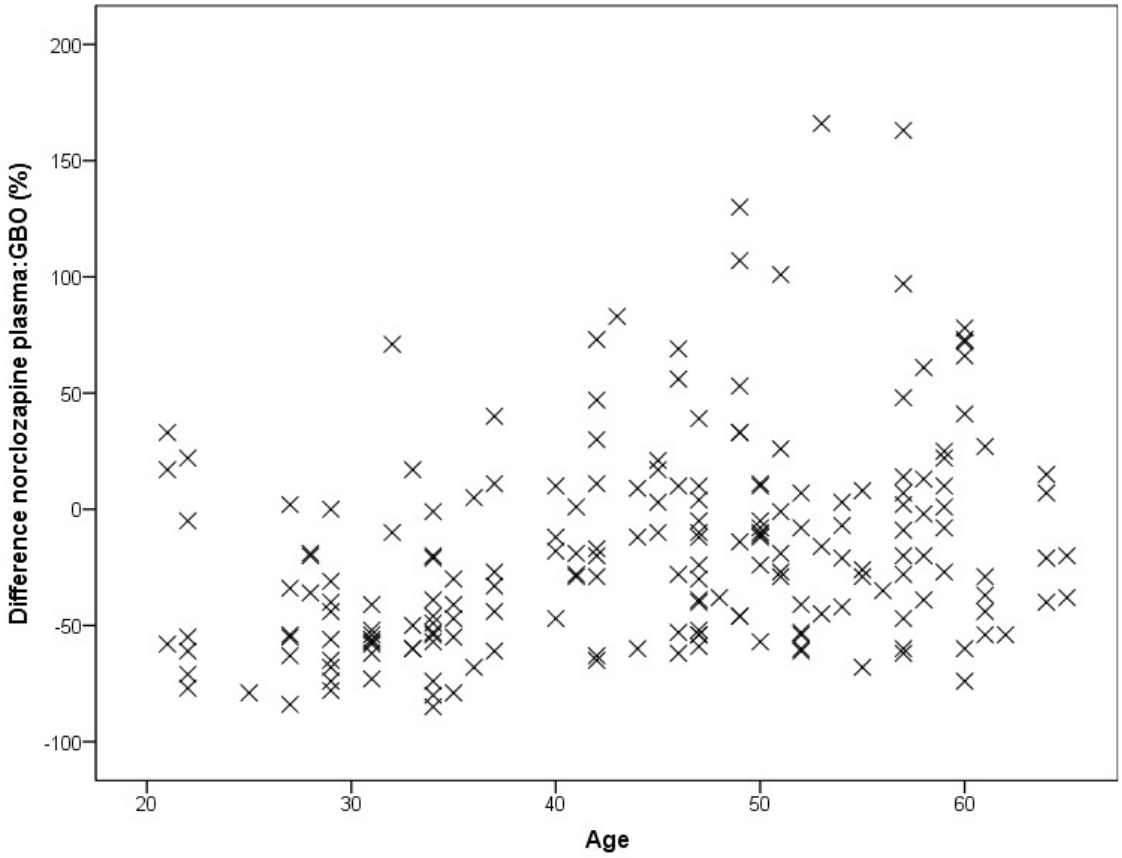


Figure 4-6 (cont.)



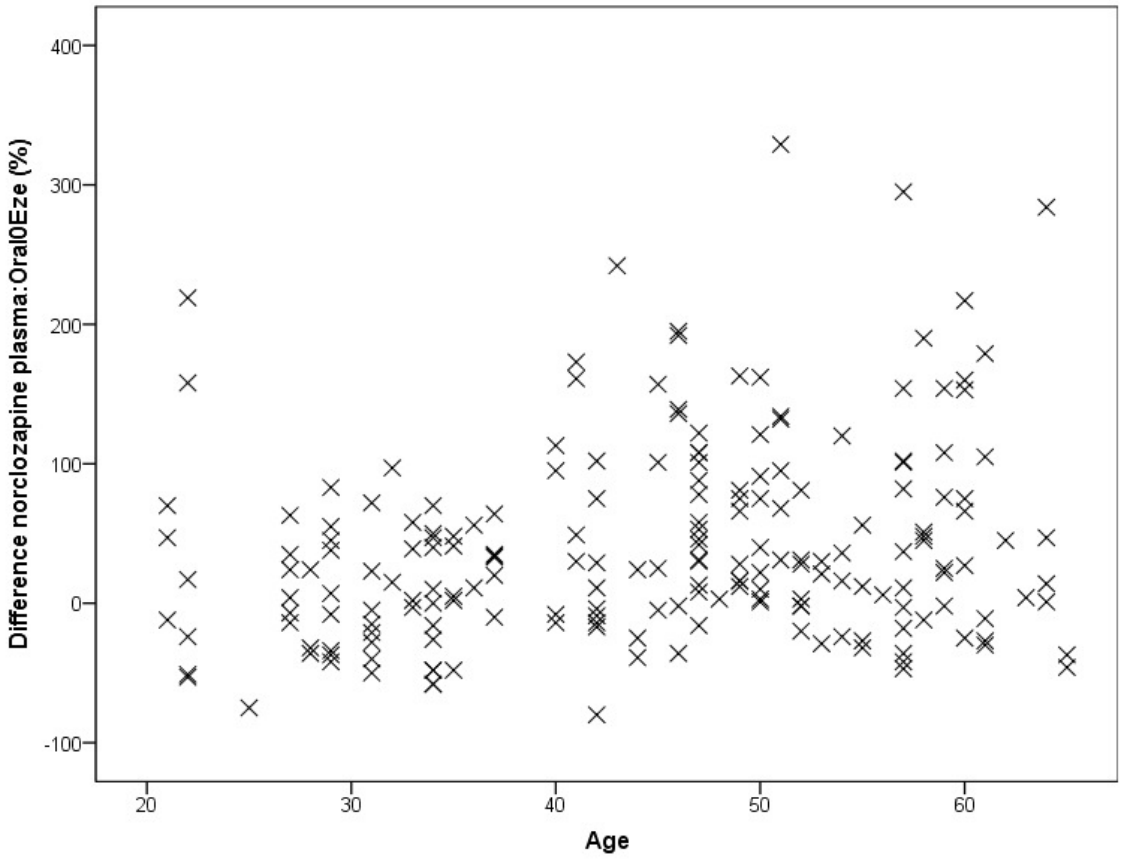
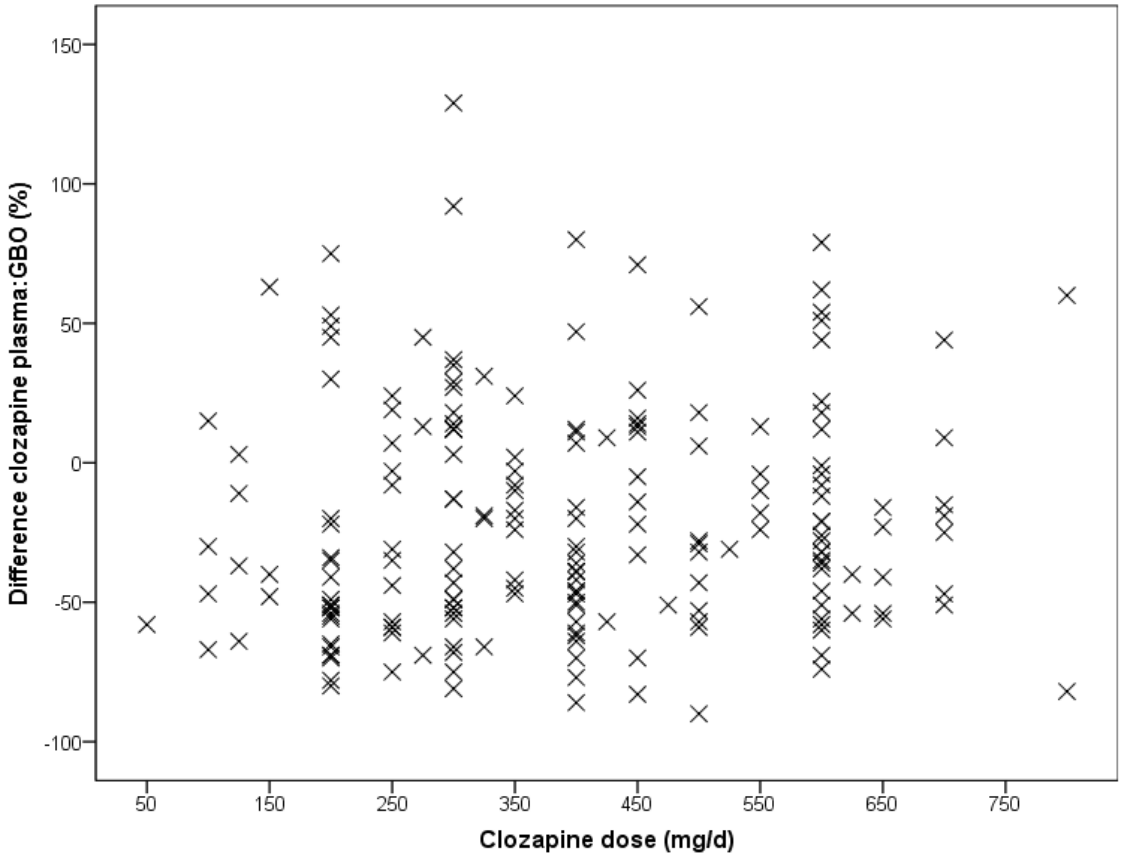


Figure 4-7: Plot of dose against difference between plasma and oral fluid clozapine and norclozapine concentrations.



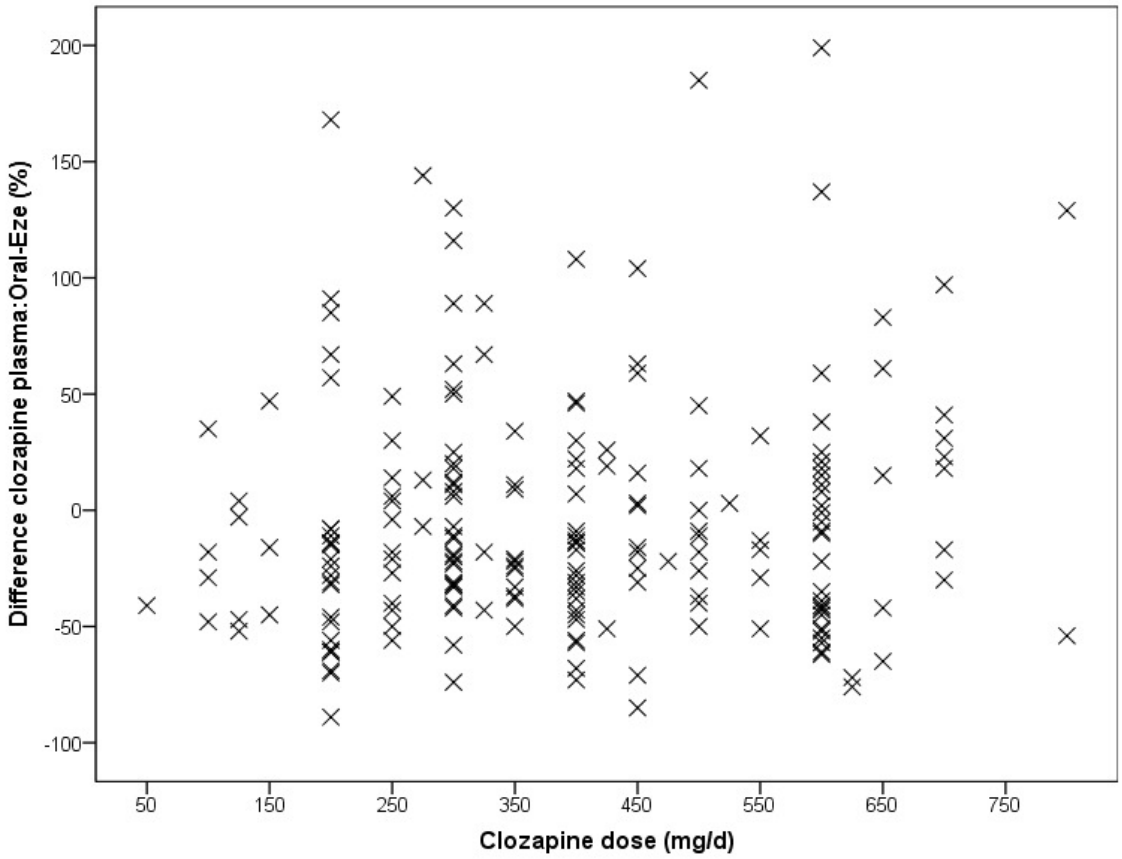
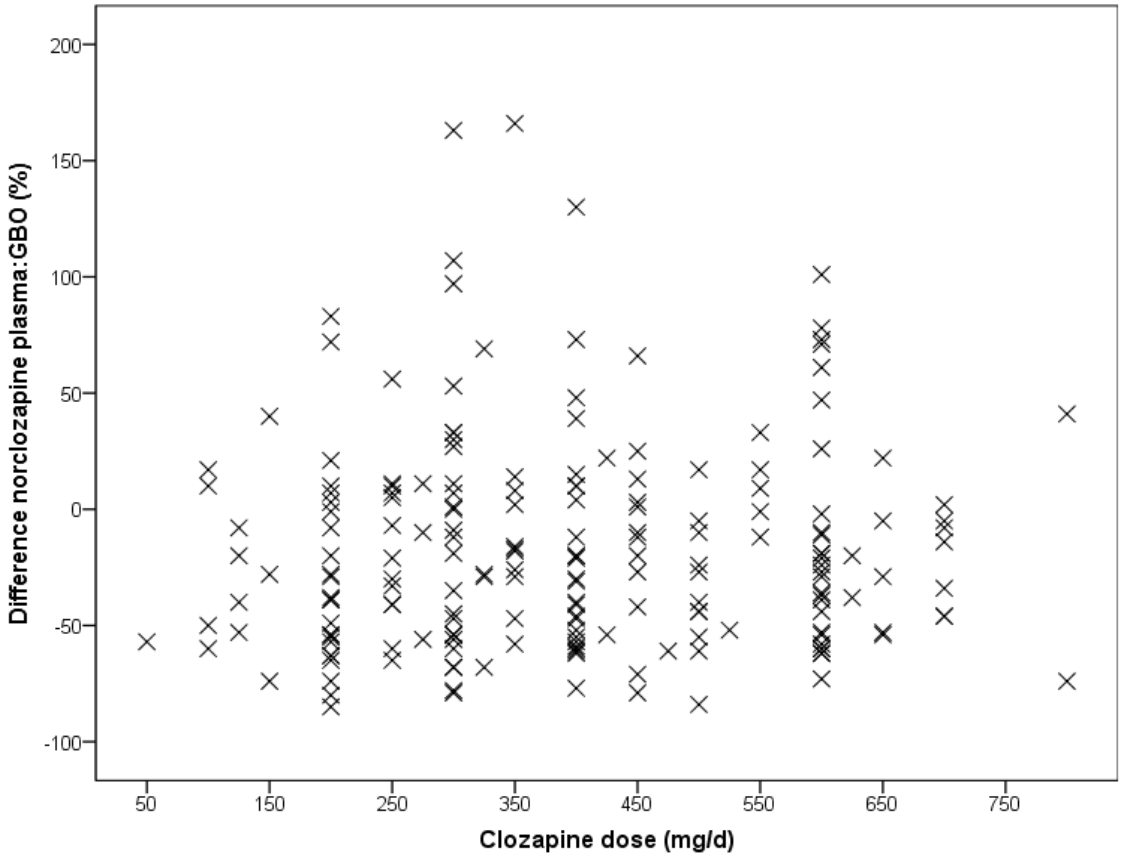


Figure 4-7 (cont.)



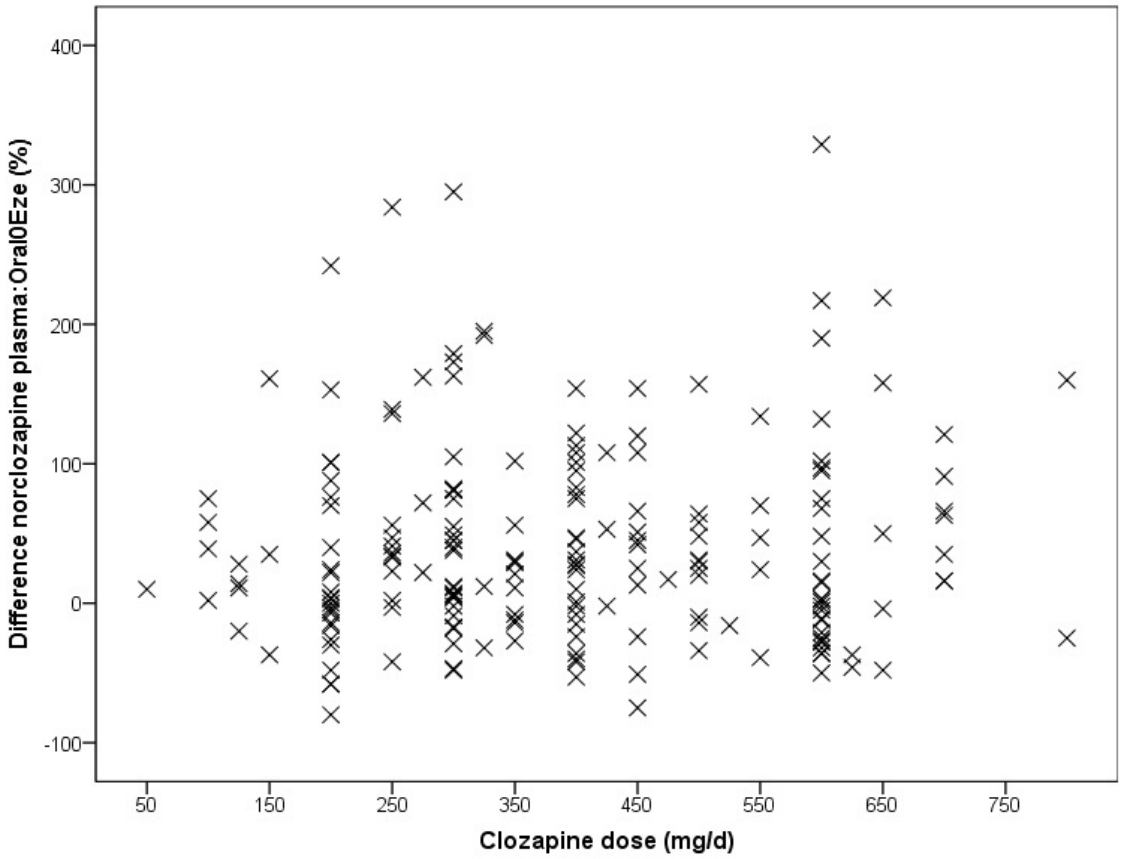
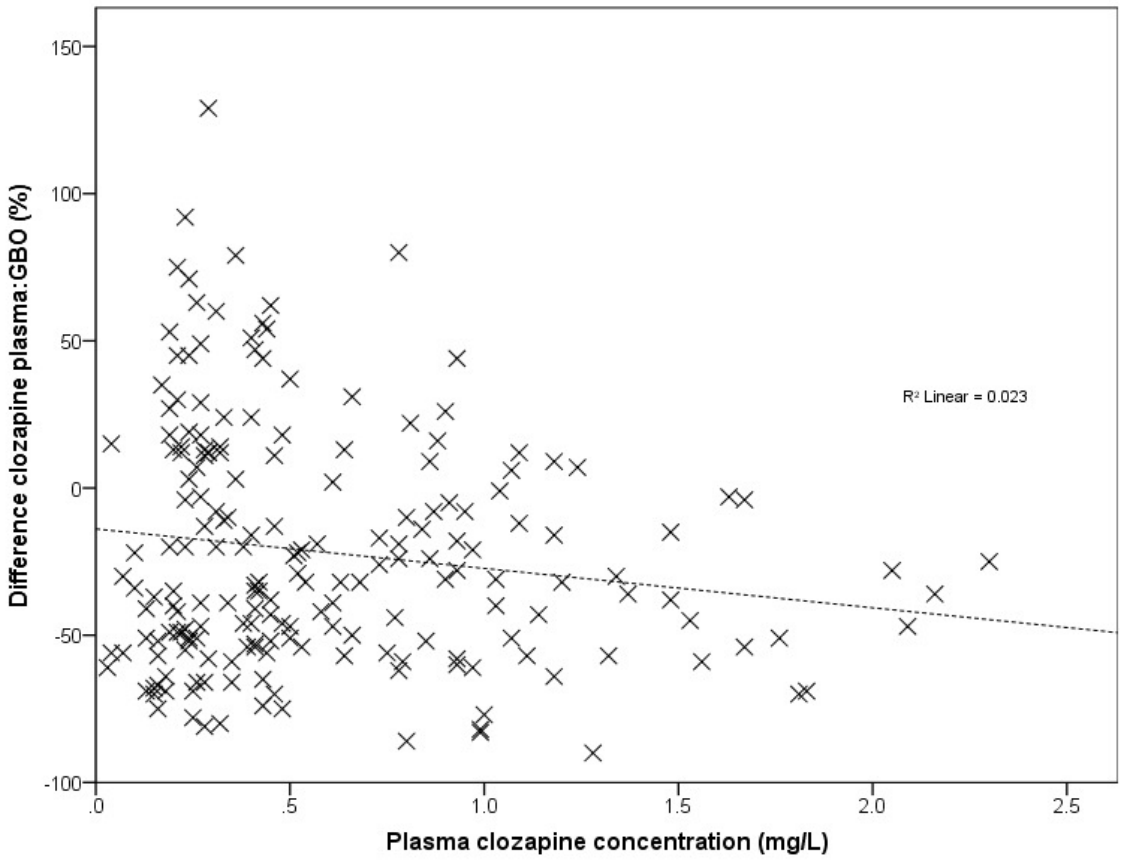


Figure 4-8: Plot of difference in plasma:oral fluid concentration and plasma concentration correlations for clozapine and norclozapine.
Trendlines shown.



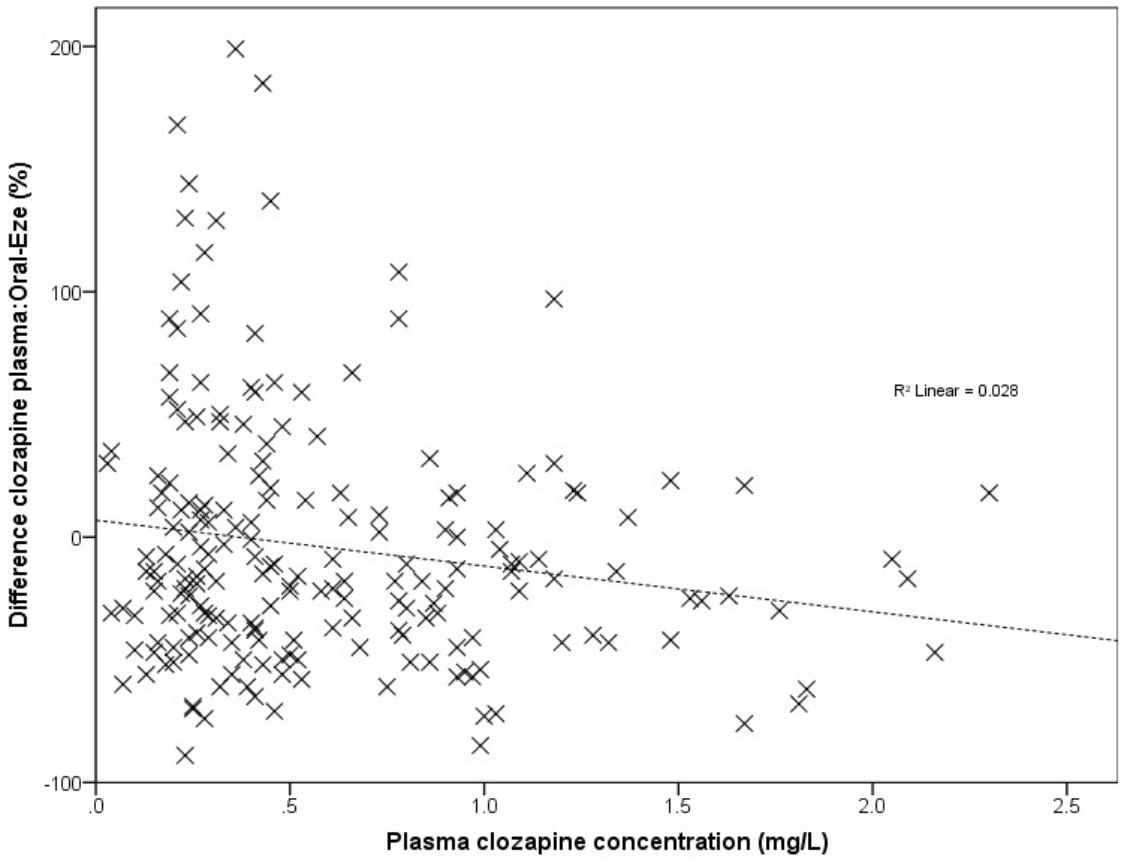
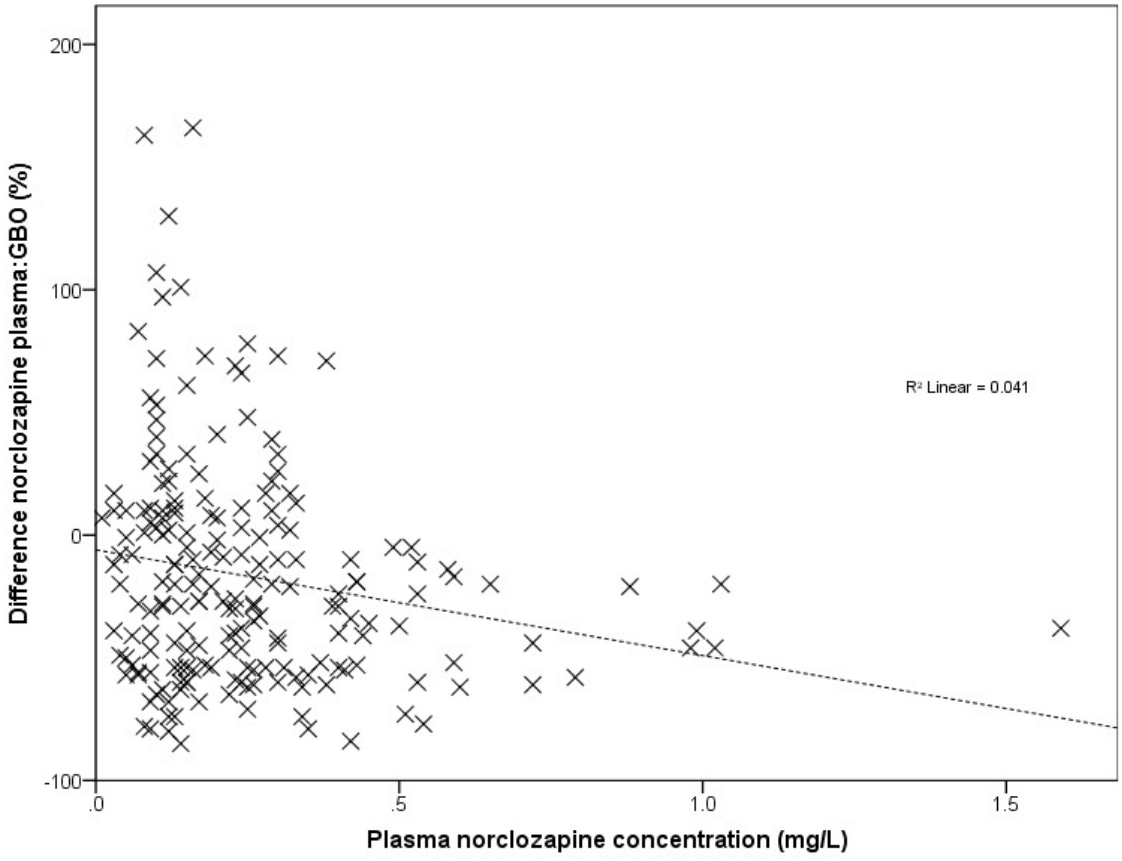
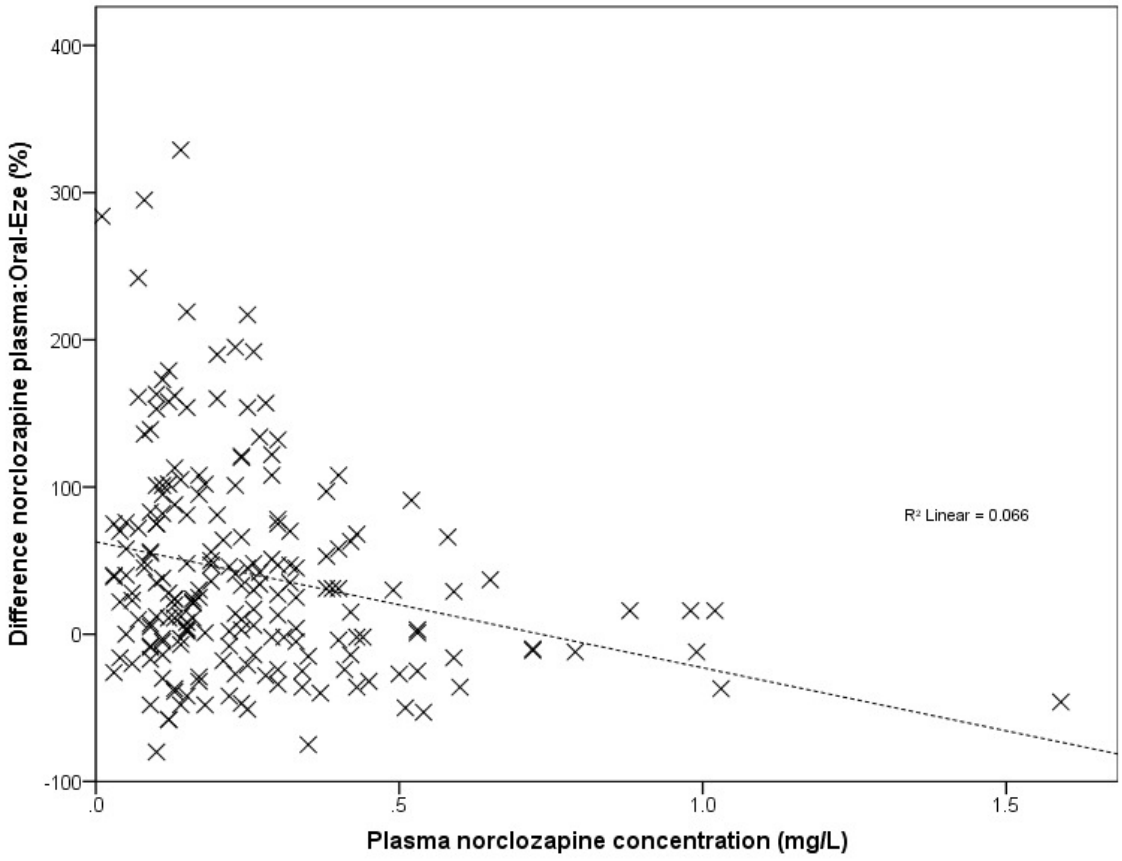


Figure 4-8 (cont.)





For both oral fluid devices, clozapine concentrations were predicted to increase by 0.05 mg/L for an increase in 10 years of age, and norclozapine concentrations by 0.03 mg/L, although the relevance of age was low in the models. Age was more relevant to the difference in plasma:oral fluid concentrations, where with an increase in age the difference in plasma:oral fluid concentrations increases, although when plotted graphically (Figure 4-6) this was due to greater degree of variability in the plasma:oral fluid concentration difference of older patients.

Clozapine dose was not highly relevant for predicting clozapine or norclozapine concentrations in either oral fluid device. Relevance was slightly higher for clozapine dose predicting the difference in analyte plasma:oral fluid concentration, but was still low as shown in the poor correlation graphically (Figure 4-7).

4.6 Amisulpride

Twenty-three patients were co-prescribed amisulpride [18 male, aged (median, range) 42 (21–64) yr and 5 female, aged 34 (29–62) yr]; 5 participants provided a single set, 17 provided two sets and 1 provided three sets, giving 42 amisulpride sample sets in total. The median (range) amisulpride dose was 400 (50–1000) mg/d. Time since last dose was known for 31 samples, median (range) 13 (11–18) hours, and dosage was split once daily in four instances, twice daily in 33 instances, and was not known for five samples.

4.6.1 Correlation between sample types

The full results are summarised in Table 4.7, which gives the mean and median amisulpride concentrations in each matrix, as well as the ratio between the matrices.

There was a clear outlier in the GBO results (Figure 4-9; GBO = 1827 µg/L, plasma = 735 µg/L, Oral-Eze = 596 µg/L), which was excluded from further analysis of the data. There was a significant difference between the median amisulpride GBO and plasma results, and between the GBO and Oral-Eze results; however, the plasma and Oral-Eze results were comparable (Figure 4-10). The correlations between the plasma and both sets of oral fluid results were similar (Table 4.8, Figure 4-11).

Table 4.7: Amisulpride concentrations in plasma and in oral fluid summary data, and between sample-type ratios.

	Concentration (µg/L)			Between sample type ratio		
	Plasma[1]	GBO[2]	Oral-Eze[1]	Plasma: GBO [2]	Plasma: Oral-Eze [1]	GBO: Oral-Eze [2]
Minimum	40	16	29	0.89	0.34	0.18
25th %	272	86	244	1.77	0.81	0.32
Median	396	153	426	2.47	1.09	0.42
75th %	541	255	604	3.42	1.32	0.57
Maximum	1338	1827	2662	4.23	1.86	1.00
Mean (SD)	446 (297)	233 (318)	501 (461)	2.57 (0.93)	1.11 (0.39)	0.47 (0.22)

[1] N = 42; [2] N = 40

Table 4.8: Correlations between plasma and oral fluid amisulpride concentrations.
* p<0.01. Excluded as outlier GBO result 1827 µg/L when corresponded to plasma 735 µg/L and Oral-Eze 596 µg/L.

	Spearman correlation		
	Plasma:GBO	Plasma:Oral-Eze	GBO:Oral-Eze
Amisulpride	0.598 *	0.882 *	0.570 *
Amisulpride excluding GBO outlier	0.867 *	-	0.870 *

Figure 4-9: Plot showing GBO outlier result for amisulpride compared to plasma and Oral-Eze results.

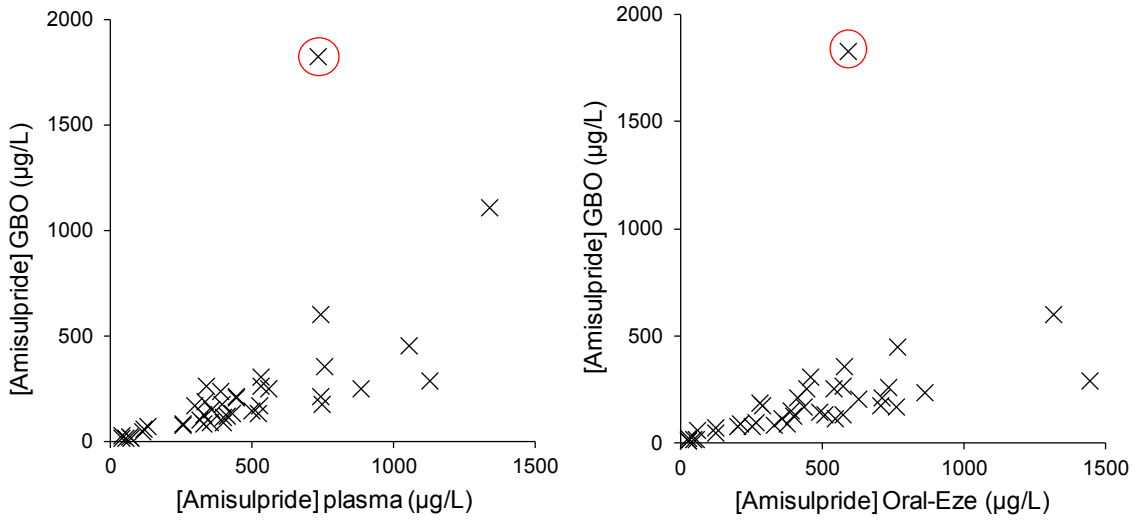


Figure 4-10: Summary patient amisulpride results comparison.
Box = median and inter-quartile range, whiskers = 95% CI; paired t-test p-value given between each dataset for each analyte. Excluded as outlier as per Figure 4-9. Plotted on log scale.

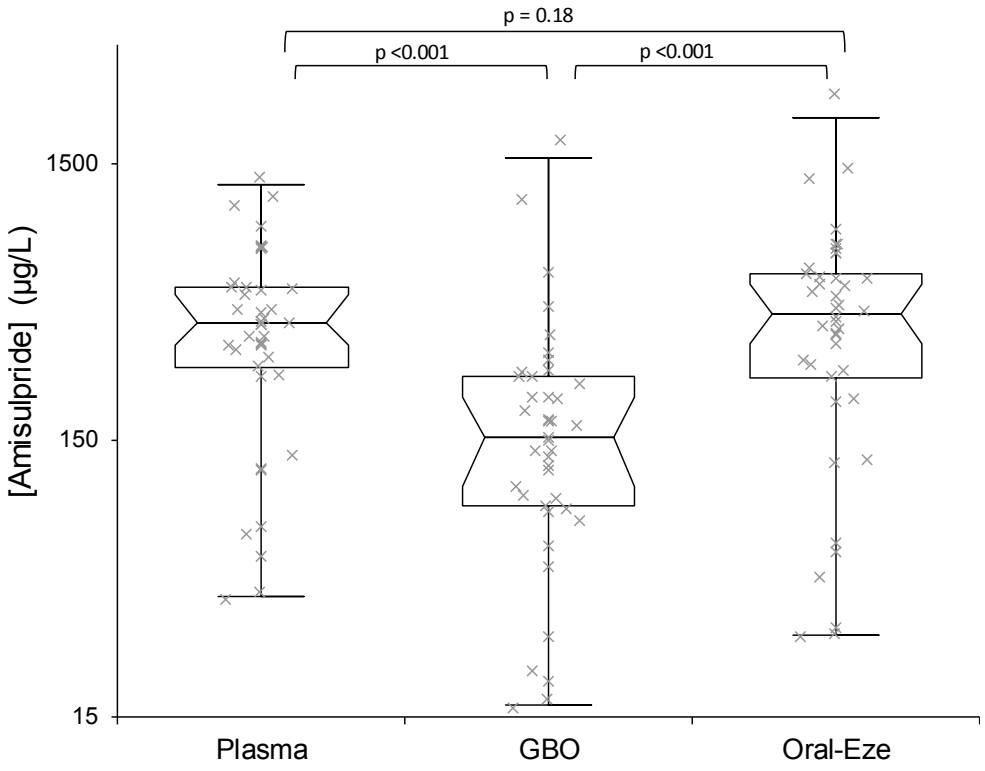


Figure 4-11: Plot of amisulpride oral fluid and plasma concentration correlations.
Excluded as outlier as per Figure 4-9. Trendlines, 95 % confidence and prediction intervals shown.

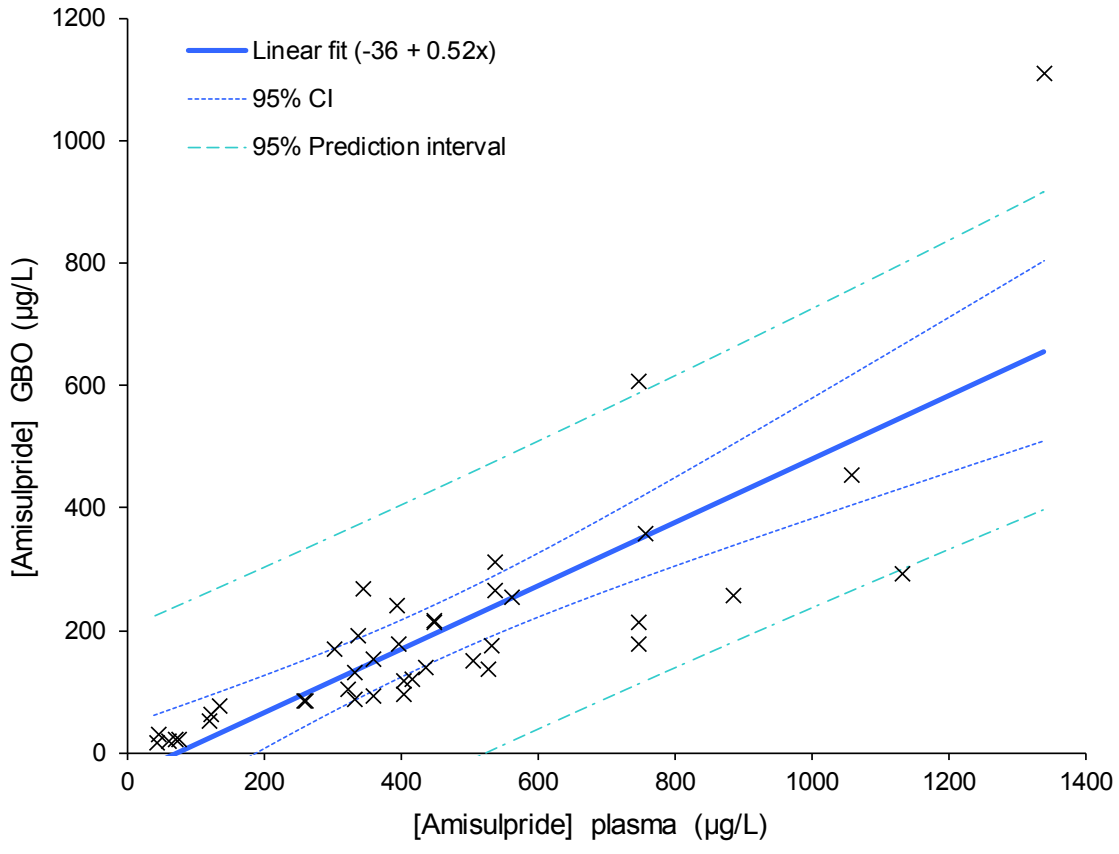
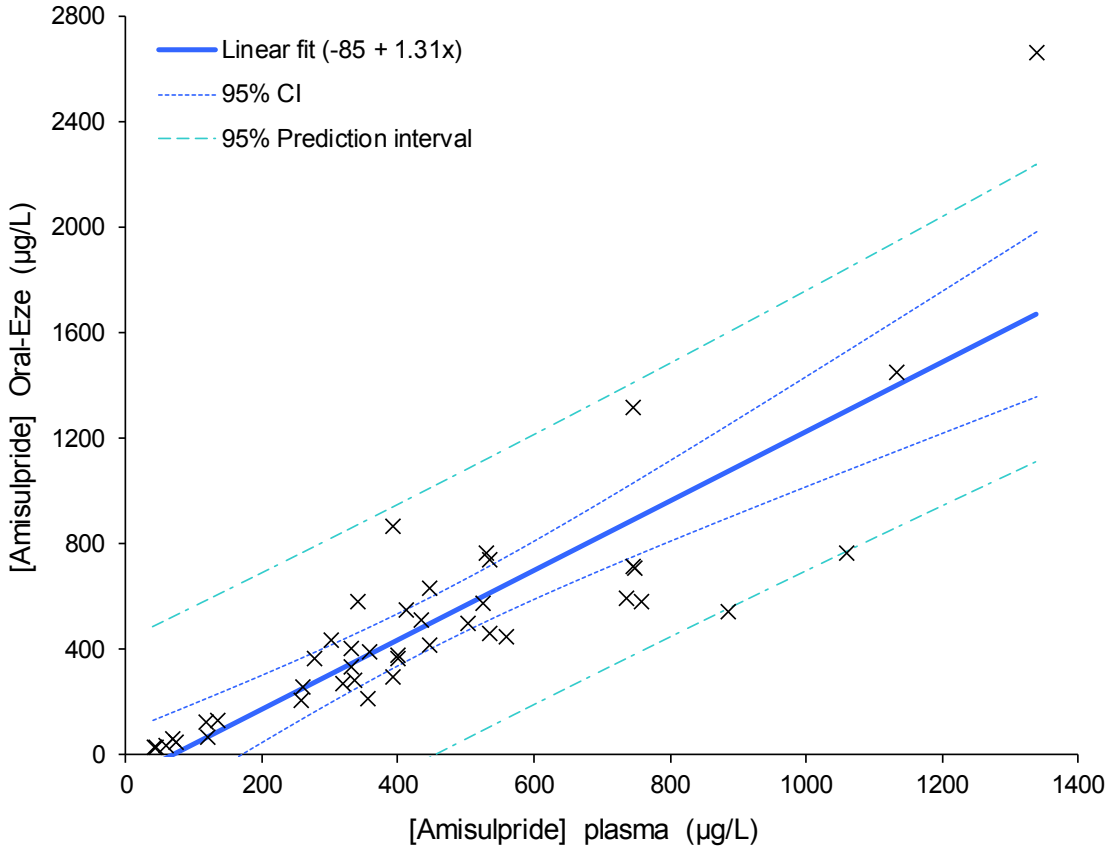
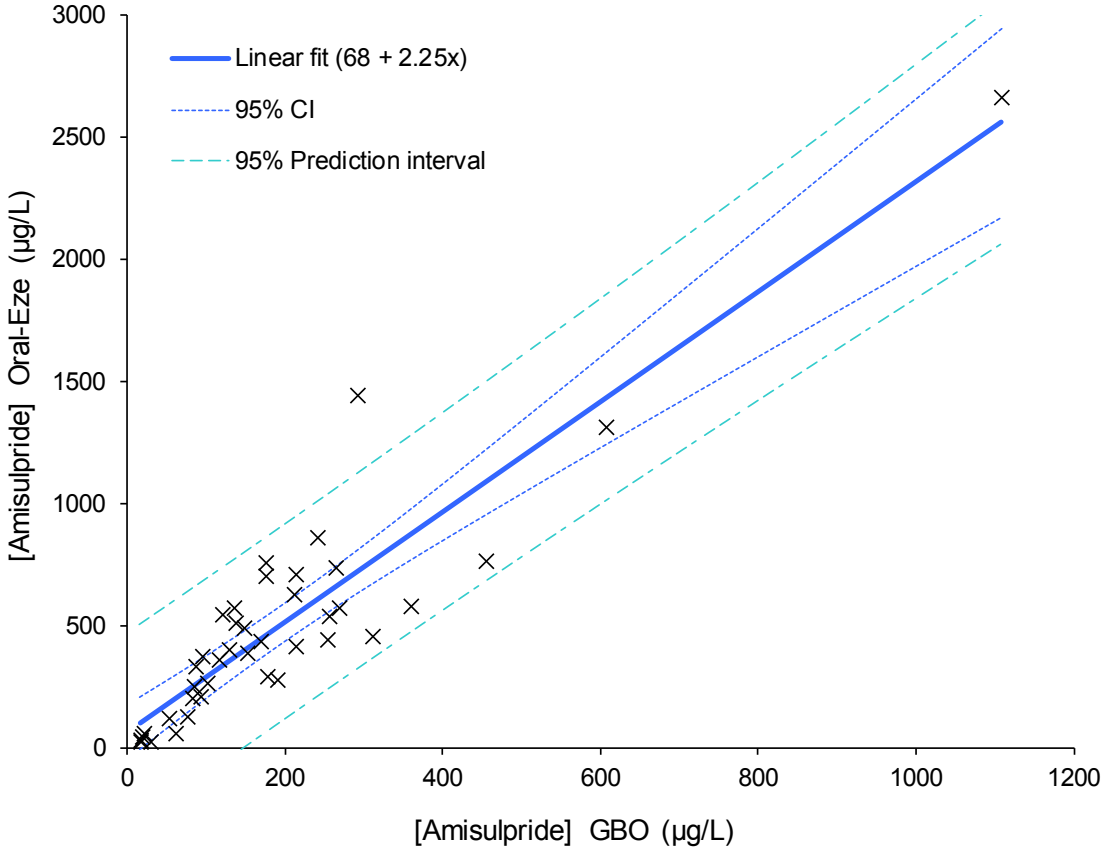


Figure 4-11 (cont.)





4.6.2 Regression analysis

Regression analyses were undertaken to investigate the relationship between amisulpride oral fluid concentrations and plasma amisulpride concentration, age, sex, amisulpride dose, time since last dose and the GBO oral fluid content (Table 4.9). As with clozapine, GBO oral fluid content was included in the regression the Oral-Eze studies. The difference in plasma compared to oral fluid amisulpride concentrations was also investigated to see if there was a proportional impact of the plasma concentration on the oral fluid result.

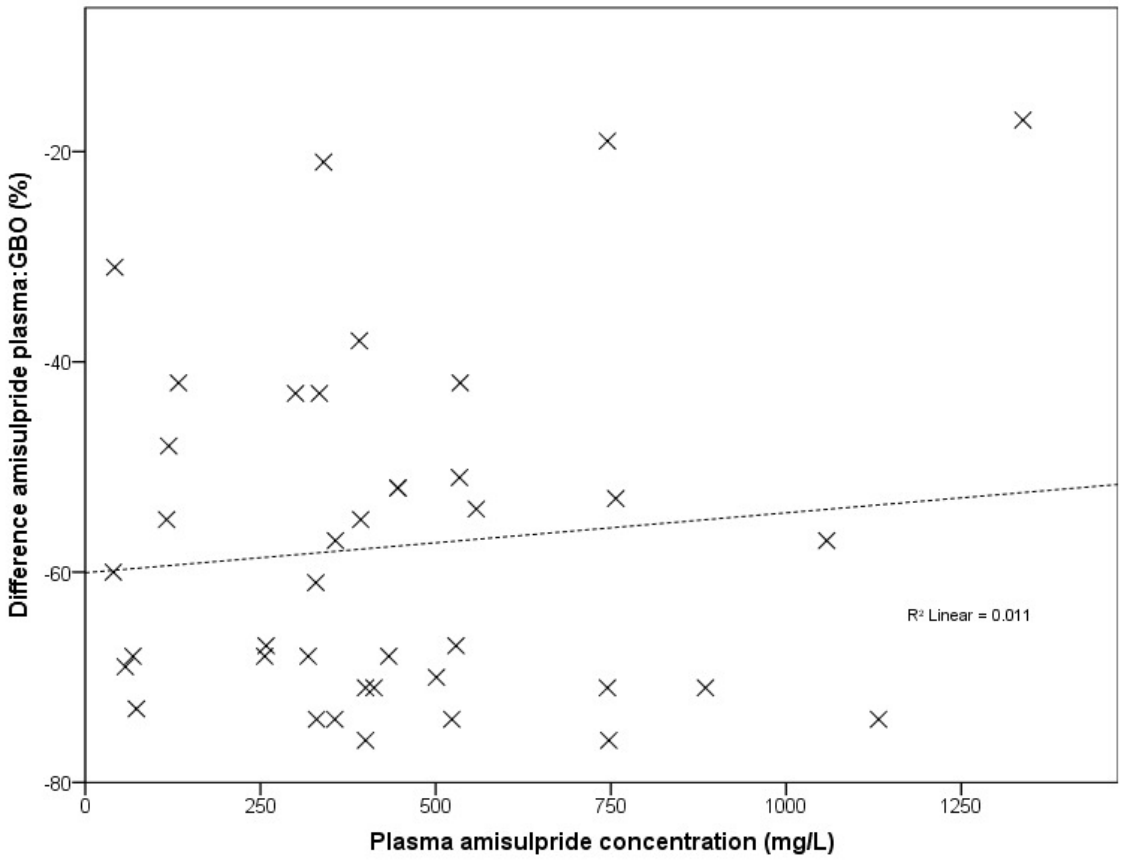
Unfortunately, due to the smaller number of results obtained for amisulpride, few models reached statistical significance. Plasma amisulpride concentration was the only significant variable for predicting oral fluid concentrations (Table 4.9); an increase in 100 µg/L plasma amisulpride concentration predicted an increase of 51 µg/L and 131 µg/L for GBO and Oral-Eze results, respectively. There were no significant variables in predicting the plasma:oral fluid concentrations difference for amisulpride, with poor correlation between plasma concentrations and the plasma:oral fluid concentration difference (Figure 4-12).

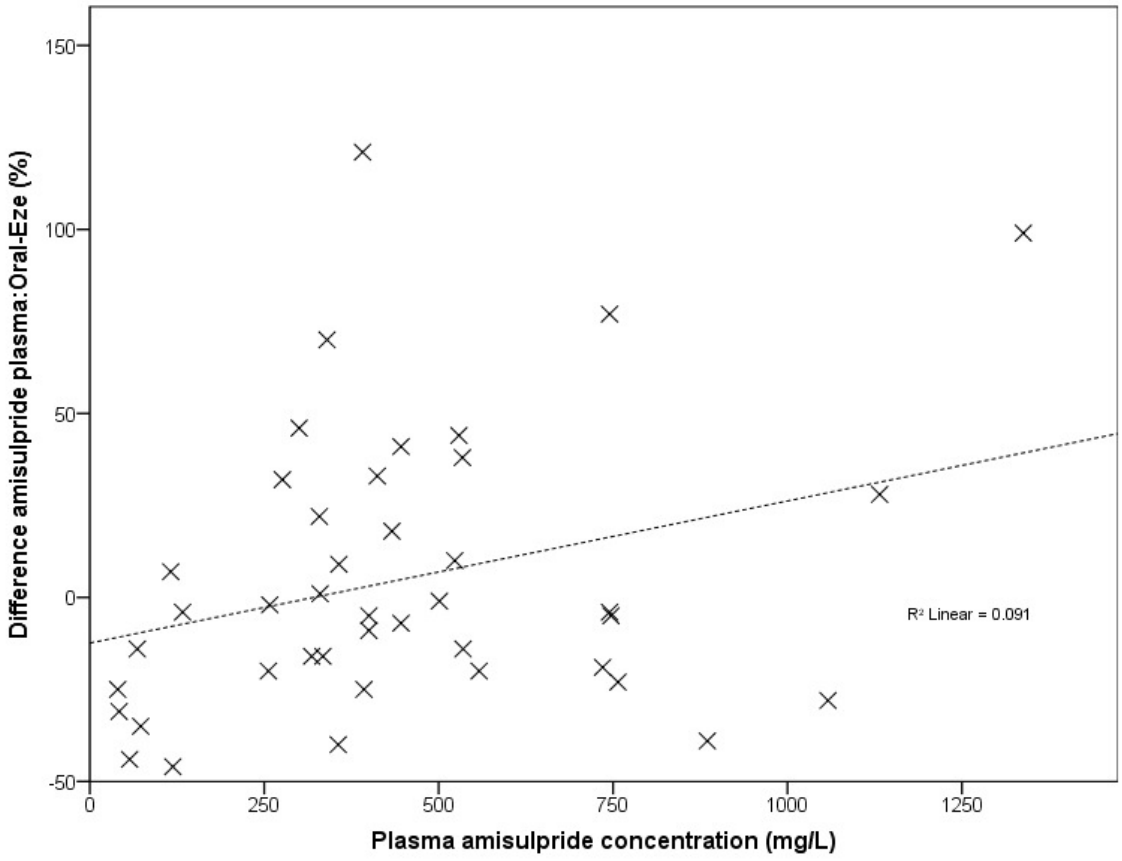
Table 4.9: Regression data for amisulpride concentration prediction.
Excluded outlier as per Figure 4-9.

Dependent variable	Model * (R, R^2)	Significant independent variables	Predictor *			
			B	95 % CI	Beta	*P* value
GBO concentration	0.81, 0.65	Plasma amisulpride concentration (µg/L)	0.52	0.39, 0.64	0.81	<0.001
		(constant)	-36.3	-102.3, 29.6	-	0.27
	Excluded GBO oral fluid content (p=041), time since dose (p=0.31), sex (p=0.99), age (p=0.65), dose (p=0.23)					
Oral-Eze concentration	0.85, 0.72	Plasma amisulpride concentration (µg/L)	1.31	1.05, 1.58	0.85	<0.001
		(constant)	-84.7	-226.2, 56.7	-	0.23
	Excluded age (p=0.97), dose (p=0.97), GBO oral fluid content (p=0.68), sex (p=0.63), time since last dose (p=0.59)					
Difference plasma:GBO	Excluded time since last dose (p=0.99), plasma concentration (p=0.84), sex (p=0.50), GBO oral fluid content (p=0.43), dose (p=0.30), age (p=0.06),					
Difference plasma: Oral-Eze	Excluded GBO oral fluid content (p=0.86), dose (p=0.67), plasma concentration (p=0.80), sex (p=0.37), time since last dose (p=0.37), age (p=0.41).					

* model and predictors explained in Section 2.2.2.1

Figure 4-12: Plot of difference in plasma:oral fluid concentration and plasma concentration correlations for amisulpride.
Trendlines shown. Excluded as outlier as per Figure 4-9.





4.7 Other analyte results

For the other analytes included within the assay, there were insufficient sample numbers to undertake statistical investigations, therefore only summaries were reported.

4.7.1 Risperidone

In total, 10 samples were obtained from 6 patients (one female aged 54, and 5 male aged 22-33) co-prescribed risperidone. A second sample was received from 4 male patients. Of the patients, the dose was stated in 4 cases as oral 3 and 4 mg/d and depot injection of 50 mg in two cases. Time since last dose was known in two cases (16 and 18 h).

Summary results (Figure 4-13) show that the GBO results were significantly different for risperidone compared to the plasma and Oral-Eze results, however the Oral-Eze results were significantly different to plasma and GBO results for 9-hydroxyrisperidone, and there was no significant difference in 9-hydroxyrisperidone MR.

4.7.2 Fluoxetine

In total, 8 samples were obtained from 4 patients (all male aged 42-57) co-prescribed fluoxetine. A second sample was received from each patient. Of the patients, the dose was stated in all cases as between 20 and 40 mg/d. Time since last dose was known in two cases (both 24 h).

Summary results (Figure 4-14) show that the fluoxetine results were significantly different between plasma and each oral fluid result, although not between the two oral fluid results. The norfluoxetine Oral-Eze results were significantly different to those from plasma and GBO samples, and for the norfluoxetine MR there was only significant difference between the GBO and Oral-Eze samples.

4.7.3 Olanzapine

In total, 8 samples were obtained from 4 patients (all male, aged 21-59) co-prescribed olanzapine. A second sample was received from 3 patients, and one provided a third sample. Of the patients, the dose was stated as between 15 and 30 mg/d, and time since last dose only known in one case (12 h).

Summary results (Figure 4-15) show there was no significant difference between the plasma and GBO concentrations, however difference was significant between Oral-Eze and the other matrices since these results were markedly higher.

4.7.4 Quetiapine

There were only two patients who were co-prescribed quetiapine, both male, aged 50 and 64 yrs; dosage was not recorded in either case. Full results are given in Table 4.10; no further analysis was possible due to the low numbers. With such small numbers, firm conclusions cannot be sought, however whilst the MR for 7-hydroxyquetiapine and *O*-desalkylquetiapine look similar between plasma and oral fluid, the MR for *N*-desalkylquetiapine was approximately 0.6 for plasma, 1 for GBO and 1.5 for Oral-Eze, suggesting a differing transfer of *N*-desalkylquetiapine compared to the other analytes into oral fluid depending on the collection device used.

Figure 4-13: Summary patient risperidone, 9-hydroxyriseridone and the metabolic ratio results comparison.
(a) risperidone, (b) 9-hydroxyrisperidone, (c) 9-hydroxyrisperidone MR ratio. Box = median and inter-quartile range, whiskers = range; paired t-test p-value given between each dataset for each analyte. 9-Hydroxyrisperidone MR calculated as 9-hydroxyrisperidone plasma concentration divided by risperidone plasma concentration.

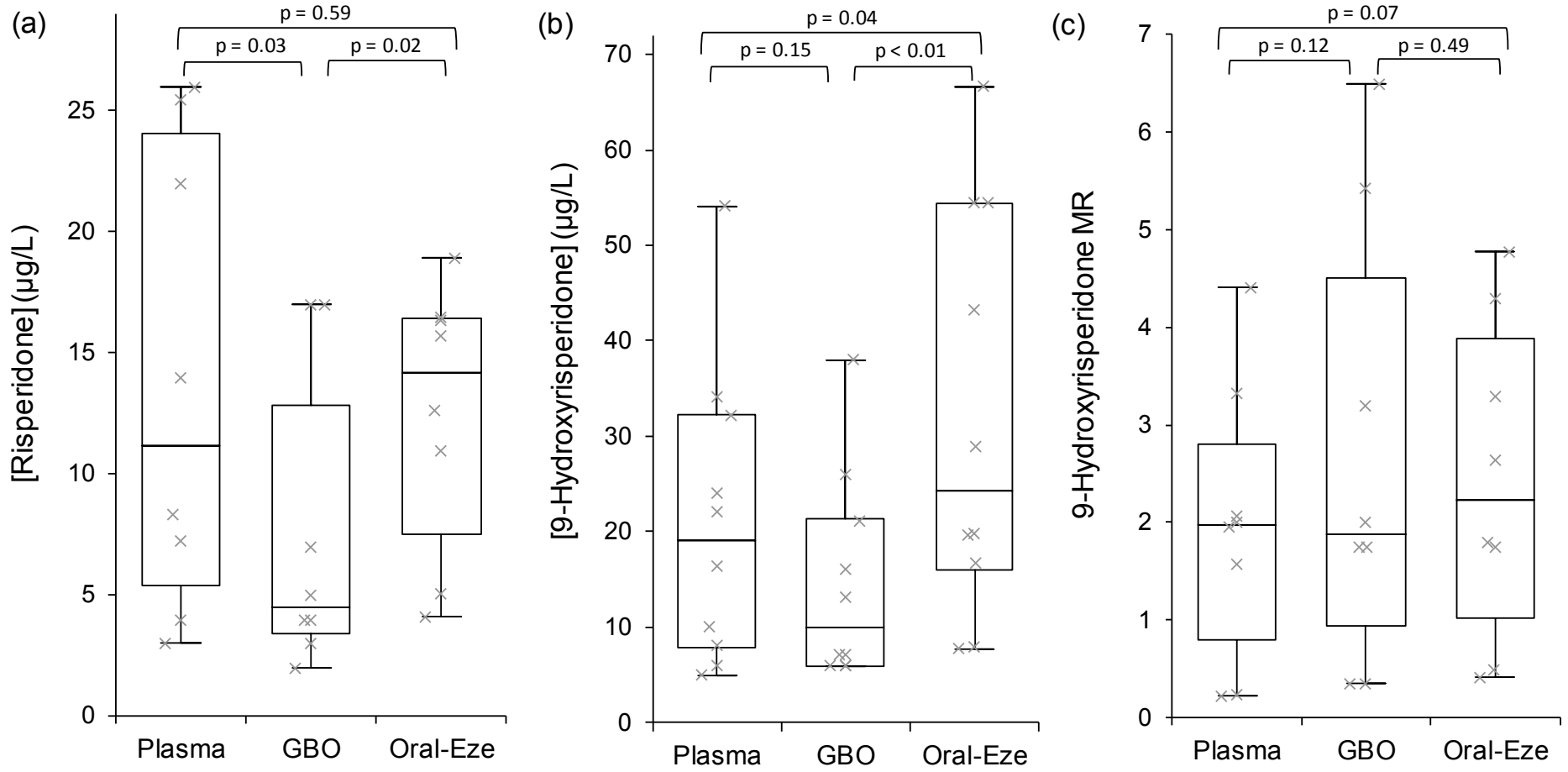


Figure 4-14: Summary patient fluoxetine, norfluoxetine and the metabolic ratio results comparison.
(a) fluoxetine, (b) norfluoxetine, (c) norfluoxetine MR ratio. Box = median and inter-quartile range, whiskers = range; paired t-test p-value given between each dataset for each analyte. Norfluoxetine MR calculated as norfluoxetine plasma concentration divided by norfluoxetine plasma concentration. Plotted on log scale.

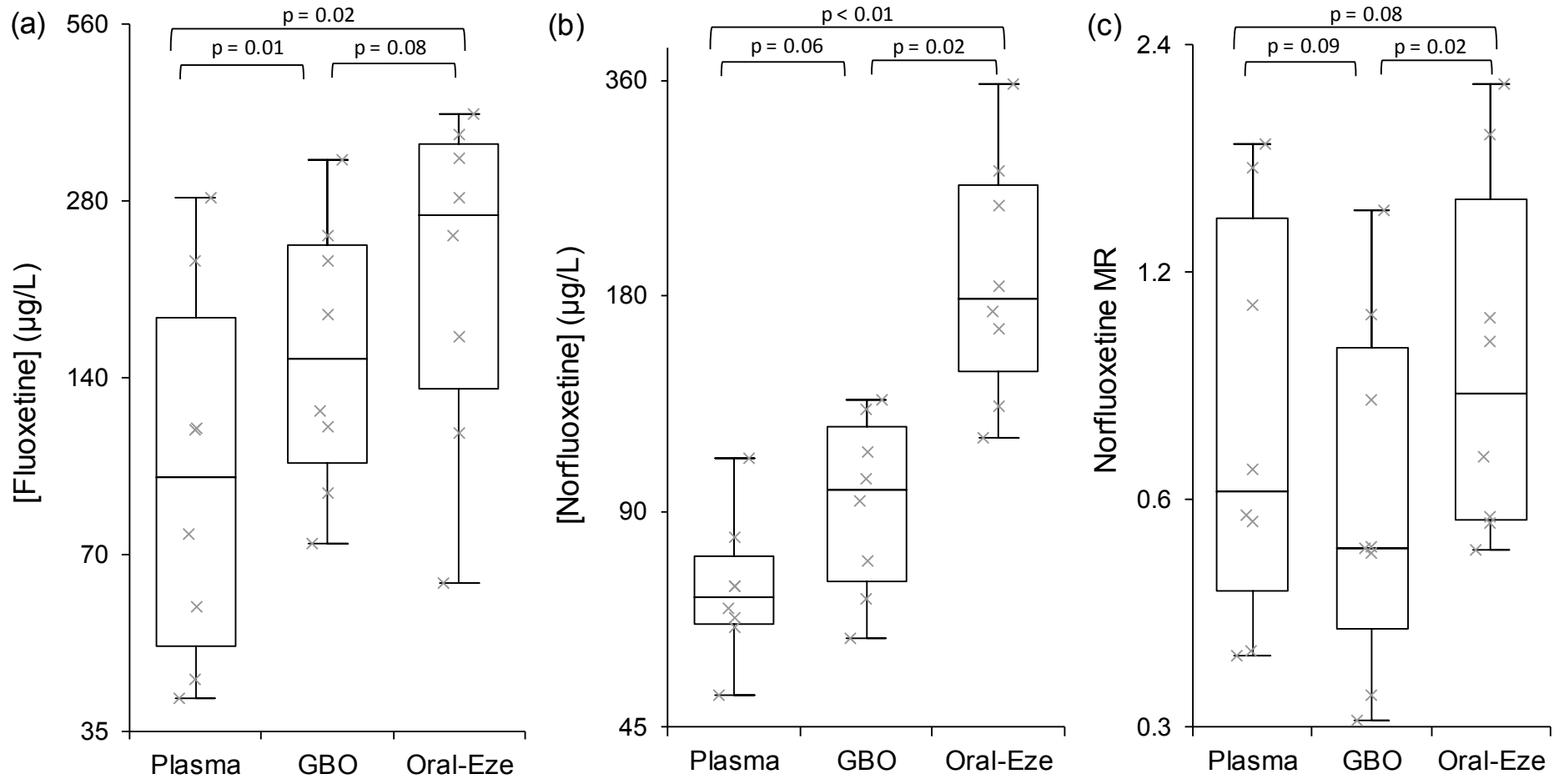


Figure 4-15: Summary patient olanzapine results comparison.
Box = median and inter-quartile range, whiskers = range; paired t-test p-value given between each dataset for each analyte. Plotted on log scale.

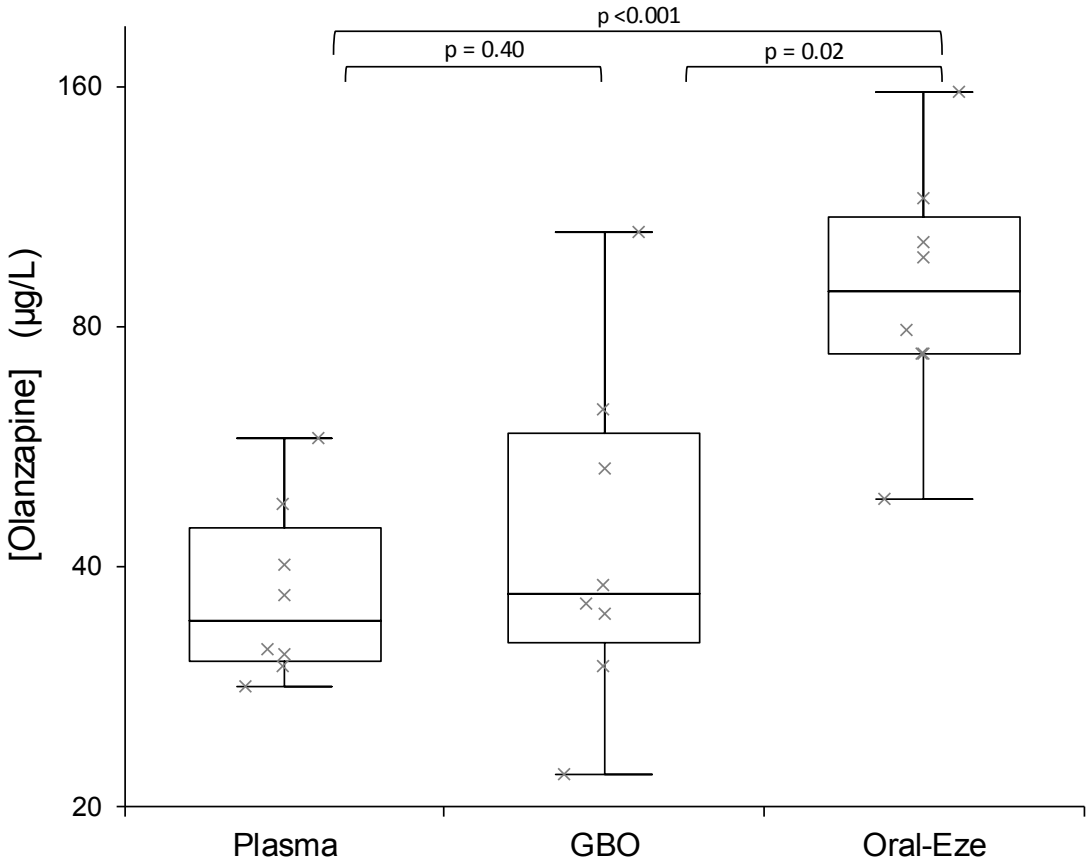


Table 4.10: Summary quetiapine patient sample set results.

Patient Sex, Age	Sample	Analyte concentration (µg/L)			
		Quetiapine	*N*-Desalkyl-quetiapine	7-Hydroxy-quetiapine	*O*-Desalkyl-quetiapine
Male, 64	Plasma	41	19	< 2	4
	GBO	23	23	3	3
	Oral-Eze	28	40	3	3
Male, 50	Plasma	22	16	< 2	3
	GBO	12	15	2	2
	Oral-Eze	11	17	2	2

4.8 Discussion

4.8.1 Application of the oral fluid devices and oral fluid content

There were some reported issues with regards to application of the devices. For the GBO device, two patients swallowed the fluid instead of holding it in the mouth. Also the accuracy of the 1 mL indicator for the Oral-Eze device was questionable; when the device was used the blue line sometimes did not move and the pad had to be removed at 10 minutes, but when taken out of the mouth suddenly jumped to higher up the pad. No specific end-of-sampling questionnaire was undertaken; were the trial to be repeated this would be something that would be of value to undertake. General comments that were reported by the clinical staff were that the patients complained about the taste of the GBO collection fluid, but overall that they preferred both processes to venepuncture.

Pre-analytical handling of the GBO samples was simple since the collection system was fully self-contained up to the point where the sample enters the laboratory from storage. However, tartrazine quantification adds another analytical requirement. As to the Oral-Eze system, the need to recover the sample after overnight refrigeration prior to storage added an extra step, but laboratory analysis was simplified by collecting a theoretical fixed volume.

Patient results were corrected for oral fluid volume in the case of the GBO system samples, and for recovery from the Oral-Eze system pad. In the case of the GBO system samples the average oral fluid content was approximately 50 % (1+1) dilution, but one sample only contained 13 % oral fluid (1+8 dilution in collection solution), which would clearly have sensitivity implications. Sensitivity was slightly poorer for the Oral-Eze samples due to the dilution (1+2) of the calibration solutions performed to reflect the dilution of the samples during sample collection, but this did not adversely affect the results. The Oral-Eze system aims to collect 1 mL oral fluid, but work with a similar device in which the collection pad was weighed before adding to the transport buffer has shown that the actual volume collected can vary dramatically (Patteet *et al*., 2016; Langel *et al*., 2008). However, this would not be practical in-clinic and we thus decided to use the device per the manufacturer's instructions to test the real-life application of the system.

As to the proportion of oral fluid collected using the GBO system, Haririan *et al*. (2016) reported values of approximately 70 ± 6 % (mean ± SD) in three groups of dental patients. However, a

mean (range) oral fluid content of 56 (39–73) % (RSD 15 %) was found in volunteers given codeine (Coucke *et al*., 2016), results much more in keeping with those obtained here, i.e. mean (range) of 51 (13–86) %. Moreover, the results from the patients who gave three different samples show that even within the same patient there can be a high degree of between collection variability in the proportion of oral fluid collected using the GBO system.

That there would be no residue remaining in the mouth after Oral-Eze sample collection and that the oral fluid could become contaminated by the GBO collection fluid was the basis of the decision to collect the Oral-Eze specimen before the GBO system specimen. The possibility that there was a hangover effect from use of the GBO system was noted by Coucke *et al*. (2016); in a volunteer study, codeine concentrations measured in oral fluid collected with the GBO system showed a better correlation with plasma codeine concentrations than in oral fluid collected with a pad-based collection device (Quantisal, Alere), particularly when Quantisal was used first. However, the correlations were still not good enough to predict plasma codeine reliably from oral fluid codeine.

The medications being investigated within this work have been reported to have some impact on salivation rates in patients; clozapine influences salivation in some patients either by enhancing saliva production, or via impairing the swallowing reflex. Amisulpride may reduce clozapine-induced hypersalivation in some instances (Kerinin *et al*., 2011). Be this as it may, there was no relationship between the volume of oral fluid collected by the GBO system, the prescribed dose, and either the plasma, or the GBO and Oral-Eze system oral fluid concentrations for any analyte.

4.8.2 Clozapine results

The clozapine and norclozapine concentrations in the GBO and Oral-Eze system samples correlated poorly with the corresponding plasma concentrations in all cases (Figure 4-2). There was also a poor correlation between the analyte concentrations between the two oral fluid samples (Figure 4-3). The results from the GBO system samples were significantly lower for both clozapine and norclozapine, although the Oral-Eze results were lower for clozapine but higher for norclozapine compared to plasma (Figure 4-4, Table 4.3). The norclozapine MR was dramatically higher in the Oral-Eze samples compared to plasma and GBO samples. These results suggest that the Oral-Eze device collects norclozapine preferentially over clozapine,

either as analyte adsorption to the pad, or as an effect of differing transfer of the analytes from plasma to saliva and into oral fluid, possibly related to plasma protein binding or analyte pK_a.

Results in oral fluid in 33 samples collected by the drool method showed a poor correlation to plasma for both clozapine and norclozapine (Fisher *et al*., 2013). For the data included herein, correlation to plasma for clozapine in both GBO and Oral-Eze sample types was slightly higher than for samples collected by the drool method, but the results were comparable or lower for norclozapine (Table 4.11). These results were surprising as the theory behind the project was that the presence of the buffer in-mouth would minimise the between-patient and between-collection variability in salivary pH, in turn improving the correlation to plasma. In addition, the earlier work using the drool method showed that there was an on-average 3.6-fold higher concentration of both clozapine and norclozapine in the plasma than in oral fluid (Table 4.11), which was not observed in the devices used in this project where the concentrations were around double in the oral fluid from both devices compared to that collected by the drool method when looked at as a ratio to plasma. This difference could be due to stimulation of salivary flow by the two collection devices which could in turn increase the rate and volume of excretion of proteins, electrolytes (Aps and Martens, 2005) and hence increase the flow into saliva of the analytes in question.

Comparison between the concentrations attained in the samples from the two different oral fluid devices show that the GBO samples attained concentrations lower than those from the Oral-Eze samples when both were corrected for oral fluid content (91 % and 63 % of GBO concentrations for clozapine and norclozapine, respectively).

Table 4.11: Comparison of clozapine correlations oral fluid to plasma

Oral fluid sample type compared to plasma	Correlation		Correlation coefficient (R^2)		Plasma:oral fluid analyte mean ratio	
	Clozapine	Norclozapine	Clozapine	Norclozapine	Clozapine	Norclozapine
GBO device	0.78	0.80	0.60	0.63	1.8	1.6
Oral-Eze device	0.77	0.76	0.59	0.58	1.4	0.9
Drool (Fisher *et al*., 2013)	0.72	0.81	0.53	0.65	3.6	3.6

Regression analysis showed that only 60-70 % of the clozapine and norclozapine oral fluid concentration variability could be predicted by the variables studied (Table 4.5 and Table 4.6). Plasma analyte concentration explained most of the variability within the model, however age and clozapine dose were also significant variables, where an increase in age and clozapine dose each predicted an increase in the oral fluid concentration. At low plasma analyte concentrations, the oral fluid concentration was lower relative to the plasma concentration (higher percentage difference plasma:oral fluid concentration; Figure 4-12), which suggests a smaller proportion of the plasma analytes are crossing into the oral fluid that could possibly be explained by a non-proportional concentration effect of analyte binding to plasma protein.

The most positive finding was that in one case a patient became non-adherent to the clozapine treatment, and that this result was identified not only in the plasma sample, but also in both oral fluid samples. The lower sensitivity due to the dilution of the analytes as collected by the oral fluid collection devices compared to plasma did not impact the identification and quantification of relevant concentrations.

4.8.3 Amisulpride results

Amisulpride is poorly (16 %) plasma protein bound (Bergemann *et al*., 2004), relatively water soluble, and less strongly basic than clozapine and thus oral fluid amisulpride concentrations in theory should be much less likely to be affected by changes in saliva flow-rate and thus pH during sample collection than those of clozapine and norclozapine. It was therefore unexpected that the correlations between plasma and oral fluid were as equally poor as those observed for clozapine and norclozapine.

The amisulpride results were particularly interesting in that the average concentration in the Oral-Eze samples was over twice that measured in the GBO samples. This despite the fact that the Oral-Eze collection time sometimes extended well beyond that expected (up to 10 min), suggesting that a greater-than-expected volume of oral fluid had been collected. The reason for this discrepancy is unclear.

4.8.4 Other analytes

Concentrations of risperidone and 9-hydroxyrisperidone were significantly lower in the GBO oral fluid samples than the Oral-Eze oral fluid samples, but the metabolic ratio was not significantly

different, suggesting that risperidone and 9-hydroxyrisperidone transfer equally into samples collected with the devices. Concentrations of fluoxetine and norfluoxetine were also lower in the GBO oral fluid samples that the Oral-Eze samples. However, norfluoxetine MR was also significantly different between the two oral fluid samples, which suggests in this case norfluoxetine transfer was less than that for fluoxetine into saliva for the GBO device.

Overall, analyte concentrations were lower in the oral fluid samples collected using the GBO device than those using the Oral-Eze device, although not all were significantly lower and the degree of difference varied between analytes. This cannot be explained, especially as the GBO device would be expected to be a more stimulated collection and therefore theoretically should increase the amount of analyte in the oral fluid. One possibility is that a greater amount of stimulation increased the proportion of some constituents of oral fluid, such as water, without increasing the transfer of analyte from plasma into saliva thereby having a dilution effect. Alternatively, it could be due to changes in the salivary pH that occur following simulation (Kato *et al*., 1993). This may explain why the analytes have different ratios between the samples collected for the GBO and Oral-Eze collection, since the analytes would transfer differentially depending on the physiochemical parameters such as pK_a and plasma protein binding.

4.8.5 Antipsychotic TDM

Patients prescribed clozapine were studied because they have blood taken routinely for haematological monitoring, and thus have a high degree of interaction with mental health professionals. In addition, clozapine and norclozapine TDM has proved valuable in assessing adherence and guiding dosage. Clozapine and norclozapine are said to be some 95 and 90 % bound, respectively, to plasma protein (Schaber *et al*., 1998), giving examples of moderately-plasma protein bound analytes. A secondary consideration was that a proportion of patients were likely to be co-prescribed amisulpride, which has different physiochemical properties to clozapine and this would provide a contrast for comparative purposes.

The results of this work have shown that there was a difference in the results obtained between two different oral fluid collection devices when compared to plasma, even when used side-by-side, for basic drugs with completely different physicochemical properties. Comparisons of the different oral fluid devices in a variety of applications have been published and overall show that there is a wide difference between device and between collection technique (Coucke *et al*., 2015; Crouch *et al*., 2008; Langel *et al*., 2008). The current work parallels the conclusion of one

paper stating the importance of fully validating each device and setting up ranges for each individual analyte (Langel *et al*., 2008), since the devices collect and stabilise analytes in different ways. The drugs investigated within this study may themselves have an impact of salivation, especially clozapine that is known to significantly induce hypersalivation. However, amisulpride may ameliorate this impact (Kreinin *et al*., 1993). No link was found either to clozapine dose or clozapine concentrations and the volume of oral fluid collected.

In addition, there appeared to be considerable differences in the transfer of the analytes from plasma to saliva and into oral fluid depending on the collection device used, even with regards to parent drugs and metabolites, therefore individual ranges and metabolic ratios would need to be identified for each individual collection device prior to application in routine use. Concentrations of some of the analytes appeared to decrease with increased salivary stimulation, possibly due to salivary pH changes (Kato *et al*., 1993) or an increase in the excretion of water acting to dilute the analytes in the oral fluid collected.

Overall the correlations observed in the study between plasma and oral fluid concentrations collected using both devices were inadequate with regards to predicting plasma concentrations for clinical TDM purposes, as has been found in other studies on antipsychotics or similar drugs (Flarakos *et al*., 2004; Horning *et al*., 1977; Saracino *et al*., 2010; Mandrioli *et al*., 2011; Patteet *et al*., 2015; Patteet *et al*., 2016). There was also no evidence of the relationship between plasma and oral fluid concentrations being affected by patient demographics, prescribed dose, or plasma concentration.

One study investigated the variability in the difference between plasma and oral fluid concentrations for eight antidepressants a week apart from one or two patients prescribed each of these medications, and found variability high for all drugs, including fluoxetine (de Castro *et al*., 2008). Venlafaxine was the least variable analyte (RSD <27 %), and was studied further in five patients. However, the study showed that although variability between samples was low in plasma, quantification of the plasma free fraction and in oral fluid remained highly variable (RSD up to 70 %), and as such they concluded that oral fluid measurement may only have benefit in special circumstances such as to investigate non-adherence.

Wille *et al*. (2009) also reported poor correlations between whole blood and oral fluid concentrations collected with the Intercept (OraSure) device of a number of common drugs of abuse such that reliable calculation of blood concentrations from oral fluid concentrations was

simply not possible. Similar findings have been reported using the Saliva-Sampler device (StatSure Diagnostic Systems) (Langel *et al*., 2014).

Even with lithium ion using a wide range of oral fluid collection techniques there is no consensus as to the validity of oral fluid as opposed to serum lithium monitoring (Langman, 2007; Shetty *et al*., 2012). Other work using investigating the relationship between plasma and oral fluid concentrations of other proteins and drugs have also been equally disappointing as regards to predicting analyte plasma concentrations (Coucke *et al*., 2016; Haririan *et al*., 2016; Kato *et al*., 1993; Nemecek *et al*., 2011)

The fact that similarly poor correlations were found here for clozapine, norclozapine, and most surprisingly also for amisulpride, under relatively controlled conditions (stable dosage, tablets only, time since last dose at least 10 hours), even using the GBO system where a known oral fluid volume in collected under defined oral cavity pH, suggests that applying these devices as per the manufacturer's instructions cannot replace plasma sampling for TDM of antipsychotics. This being said, if complete non-adherence is suspected oral fluid analysis may give clinically relevant information.

Further investigation into the variability of analyte recovery from the pad, as discussed in Chapter 3, will gather more information into whether the impact of this variable can be minimised in future modifications to the device. In addition, whether the use of a pH 7.4-buffered in mouth collection system would yield better results can only be speculation at this stage, but this may be the next step forward in attempting to develop an oral fluid assay for antipsychotic TDM.

The main positive finding from the present study was that a patient who had become non-adherent to clozapine was identified by analysis of oral fluid collected using both systems. Although non-adherence to clozapine is fortunately quite rare, non-adherence to other drugs such as β-blockers is a real clinical problem (Corrêa *et al*., 2016). However, in contrast to testing for either non-adherence to prescribed therapy, or illicit drug use where detection and qualitative identification is all that is needed, information to assess possible partial adherence and guide dosage is simply not provided by qualitative testing. In conclusion, the results emphasise that oral fluid collected using either the GBO system, or the Oral-Eze system cannot be used for quantitative TDM of the analytes studied.

Chapter 5. Conclusions

Therapeutic drug monitoring can be a vital part of optimising drug therapy, and its use in treatment with some antipsychotic drugs has been established.

5.1 Quetiapine metabolite TDM

TDM is used frequently in assessing adherence to quetiapine, however there is yet to be a reference range established for plasma quetiapine concentrations associated with clinical response. One possible reason for this is that the plasma half-life of quetiapine is quite short, and in addition at least one of the quetiapine metabolites, *N*-desalkylquetiapine, is believed to be pharmacologically active in depression. As such, inclusion of some or all plasma quetiapine metabolites in TDM may be necessary for a clinical reference range, or at least to provide further information for investigating quetiapine exposure.

Plasma *N*-desalkylquetiapine concentrations attained during routine therapy in this study match those reported by other studies. In the case of both *O*-desalkylquetiapine and 7-hydroxyquetiapine there are no published reports of the plasma concentrations of these metabolites attained during routine quetiapine treatment, except for the pilot study of small samples numbers (Fisher *et al.*, 2012A). The work presented herein includes many more patient samples, and dose-dependent tables for the plasma concentrations of quetiapine and its metabolites attained at difference prescribed doses have been presented to aid interpretation of future results.

Investigating the relationships between quetiapine and its metabolites showed that *O*-desalkylquetiapine shows a strong relationship to plasma quetiapine that is not matched by either other metabolite. However, 7-hydroxyquetiapine was the only metabolite that showed a significant relationship to age. Formulation only affected *N*-desalkylquetiapine MR and *O*-desalkylquetiapine C/D, whereas sex affected only the C/D for both *N*-desalkylquetiapine and *O*-desalkylquetiapine. As such, it appears that each metabolite is affected differently through the activity of different enzymes and transporters, and investigating plasma concentrations of each metabolite alongside quetiapine represents additional information that can benefit in interpreting quetiapine results.

The audit undertaken herein used results from samples sent for routine plasma quetiapine TDM, therefore there will have been a variety of reasons why samples were sent for analysis.

Thus, the data presented here may include cases of poor adherence to the medication regimes and hence the results cannot be taken as plasma concentrations associated with a good clinical response and minimal side effects.

5.2 Oral fluid for antipsychotic TDM

Use of less invasive sampling procedures may enhance the use of TDM for antipsychotics; therefore the role of oral fluid sampling was investigated. A pilot study had compared the plasma concentrations attained in patients routinely prescribed these medications to concentrations attained in oral fluid collected via the drool method (unstimulated oral fluid; Fisher *et al.*, 2012A). The results showed that the relationship between plasma and unstimulated oral fluid concentrations were poor for the drugs investigated, including clozapine. One possible reason for this poor relationship was the variability in the pH of the oral fluid, as contributed to by salivary pH and affected by the degree of stimulation and between-patient variability in the resting pH of the oral cavity. Utilising a device that stimulates salivary flow could in theory reduce between-patient and between-sample variability in oral fluid pH, and thus could improve the relationship between plasma and oral fluid concentrations, and in turn enable oral fluid results to be interpreted against the established plasma reference ranges.

Two oral fluid collection devices were selected to test this theory; the GBO collection system which uses a buffered collection fluid held in the mouth that contains a dye from which the total volume of oral fluid collected can be calculated, and the Oral-Eze device which uses an untreated pad held against the cheek until the indicator shows 1 mL of oral fluid has been collected. Holding the pad in the mouth lightly stimulated salivary flow, and post-collection the pad is stored in a buffer solution to allow the adsorbed analytes to equilibrate with the buffer, before this solution is collected and analysed. Variability in the relationship to plasma concentration was consistent between the two oral fluid devices. Concerns were raised regarding accuracy of the one mL collection volume for the Oral-Eze device. However, if this was inaccurate then it would be suggested that the relationship to plasma should be improved for the GBO device for which the volume of oral fluid is accurately calculated. As such, it appears that there is an inherent variability in the results that is not reliant purely on the volume collected, and not improved by collecting oral fluid when the oral cavity pH is buffered to an acidic pH. Reassessment of the variability in analyte adsorption/recovery from the pad to a

greater degree according to the proposal discussed in Chapter 3 may shed more light on the impact of this variable.

Both devices stored the collected oral fluid within an acidic buffer and this was shown to improve olanzapine stability compared to previous work using unstimulated oral fluid alone, where olanzapine was not stable for even a few days (Fisher *et al.*, 2013C).

Analytical considerations of using the oral fluid collection devices included, for the GBO device, the need to quantify the tartrazine dye concentration, and consideration of an observed up to 1+8 dilution of the oral fluid. For the Oral-Eze samples there was no need for an extra quantification step since the device assumes of fixed 1 mL volume collection, however consideration needed to be made for the 1+2 dilution of oral fluid into the collection buffer, and it was found that the 1 mL indicator did not appear to flow consistently.

Although there was no formal questionnaire to present patient opinion, it was reported that both devices were found to be well tolerated by the patients and preferred to venepuncture, although there was some dislike of the taste of the GBO device and confusion over collection process. Further investigation into patient preference between devices would be useful in future studies.

Analysing the 200 sample sets that were collected from patients showed that the correlation between the plasma and oral fluid concentrations for clozapine and norclozapine was not improved compared to those results from the unstimulated (drool) samples. In addition, there was no significant degree of predictability as regards the oral fluid analyte concentrations with regards to the variables considered, such as patient demographics and prescribed dose.

It was found that for many of the medications the analyte concentrations were significantly lower in the GBO samples than in the Oral-Eze samples. This was in some ways unexpected since the GBO device utilises a buffered liquid held in the mouth which theoretically would trap the basic drugs within the acidic fluid, however this observed result could simply be a feature of the stimulation of salivary flow. The presence of the citric acid acidic buffer in the mouth would stimulate salivary flow, and therefore increase the amount of analyte crossing into the oral fluid. However, one possibility is that the increased salivary flow, which is known to affect saliva pH, affected the transfer of the analytes from plasma across into saliva and decrease the ratio of analyte compared to other salivary components such as water and proteins. Device-specific reference ranges may be required due to the analyte-specific variability in samples collected

using different oral fluid collection devices. In addition, consideration must be made to ensure limits of detection match the dilution applicable to the samples collected using different devices.

The GBO oral fluid collection device trialled in this study relied upon salivary stimulation from holding an acidic buffered solution in the mouth. Using this buffer did not improve the correlation of analyte plasma concentrations to the oral fluid concentrations compared to a non-buffered lightly stimulating collection device (Oral-Eze). However, analyte-specific variability between the results of the two devices was observed. As such, this leaves a possibility that modifying the buffer applied may yet improve the correlation. Plasma pH is controlled by homeostasis to between pH 7.35 and 7.45, whereas salivary pH is dependent on a number of factors including salivary stimulation (whether physical or psychological) that means the pH of the oral cavity is so variable. Therefore, whether buffering the oral cavity to match the pH of plasma (~pH 7.4) would remove the influence of the degree of stimulation should be tested.

In one case, a patient prescribed clozapine became non-adherent to the medication regime and this was identified not only in the plasma sample, but also in both oral fluid samples. As such, whilst the application of oral fluid to interpret TDM against the plasma concentration reference ranges will not be possible based on the data presented here, there may be a role of oral fluid in special circumstances such as detecting non-adherence, not only to other centrally-acting drugs but also to β-blockers, for example (Corrêa *et al*., 2016). Additionally, changes to the devices or collection process as discussed may enable greater reduction in the variability seen.

5.3 Future opportunities for antipsychotic TDM

The value of including metabolites within plasma TDM of quetiapine has been illustrated, and the dose-dependent metabolite concentrations attained in patients prescribed quetiapine may enable better interpretation of future TDM results. Investigation of plasma metabolite concentrations attained in patients responding to quetiapine in the treatment of different illnesses would enable much greater understanding of the role of quetiapine metabolites in quetiapine therapy. This may allow development of a treatment schedule that is personalised to the individual based on the diagnosis and measured plasma concentrations, rather than being driven purely by recommended dosage regimens.

This study shows that there is currently no way to use oral fluid collected using the existing devices (as presented and following the described procedures) reliably to replace plasma TDM

of antipsychotics, and likely of other basic drugs, although there may be specific applications where oral fluid sampling could be used. Modifications to the collection procedure, whether looking at ways to reduce the variability in analyte recovery from the Oral-Eze pad, introducing a different collection process to make the collection more accurate to 1 mL, or changing the pH of the buffered in-mouth collection solution to pH 7.4, for example (as discussed in Chapter 4), may enable development of oral fluid TDM. Clinically, the results obtained by oral fluid measurements at this stage would not be able to guide dose modifications, although there may be a role when investigating non-adherence.

Options other than oral fluid to reduce the need for venepuncture include using capillary blood finger-prick samples and sweat. Recent work has shown that there was significant correlation between venous and capillary plasma concentrations of SGAs (Remmerie *et al.*, 2016). However, whilst the relationship was significant, as with the oral fluid work presented herein, the correlation was not high and there appeared to be large outliers in the data that appeared to be at concentrations twice those predicted by the trendline. In addition, the conditions for collection of the sample were not thoroughly described, and it may not be possible to replicate the same techniques in a real-life clinical environment.

Application of capillary blood sampling for antipsychotic TDM has the issue that the capillary sample will be generally be whole blood rather than plasma, and therefore the analyte whole blood:plasma distribution must be taken into account. Capillary blood is not an equivalent matrix to venous blood, and in addition during sampling there will likely be contamination from tissue fluid and sweat. Applying finger-prick blood to paper to form a dried-blood spot is well established in some fields, such as new-born screening. However, the volume collecting and spreading of a dried blood spot is affected by many variables including haematocrit and whether the finger is squeezed during collection (Enderle *et al.*, 2016). As such, qualitative screening is much more suitable to this collection method than quantitative analysis.

Micro-sampling techniques that collect a fixed volume of finger-prick blood are increasingly being used, and have been applied to different fields of TDM including a positive finding for hydroxychloroquine (Qu *et al.*, 2017). This was proposed as an advance since it removes the limitations of dried-blood spots such as sample inhomogeneity and haematocrit, whilst still retaining the advantages such as at-home sampling and reduced sample handling logistics.

Other options for alternative matrices include sweat and breath, although in general these have qualitative applications. Analysis of sweat relies on knowledge of the transfer of drugs from plasma into the sweat ducts and rates of excretion. Sweat analysis is used for analysis of chloride content in sweat to diagnose cystic fibrosis, although there are limitations based on establishing the sample volume, the rate of excretion and external contamination (Velghe *et al.*, 2016).

In general, breath analysis has not been implemented widely, as limitations include logistical issues in the storage and collection of exhaled air, although applications include investigation of asthma and chronic obstructive pulmonary disease (Velghe *et al.*, 2016). Roadside testing is a convenient means of rapid identification of exposure to illicit substances that may impair driving performance. Recent UK legislation has imposed blood limits for some commonly abused drugs such as amphetamines and cocaine analogous to those in force for blood ethanol (Department of Transport, 2013), which can be measured indirectly and non-invasively by use of breath ethanol. By extension there is also interest in the use of quantitative measurements of common drugs of abuse in oral fluid to obviate the need for venepuncture (Gjerde *et al.*, 2015). However, the nature of the sample collection device used may affect limits of detection and, based on the work on antipsychotics described here, may not apply a consistent relationship between the blood and oral fluid concentrations of common drugs of abuse given that the physicochemical properties of all the compounds are so similar.

With advancements in sampling techniques, there is a risk that analytical methods may not be suitable for the increasing requirement for sensitivity and selectivity. Use of LC-MS/MS, as shown in Chapter 3, can meet the requirements for identifying many relevant analytes from a single sample. The speed at which the results are available and can be acted upon to optimise patient therapy is an important aspect of TDM. Advancements have enabled sample extraction to be semi-automated (Couchman *et al.*, 2016), and fast-LC techniques have been developed to analyse multiple drugs and metabolites within a 1-minute gradient analysis (Couchman *et al.*, 2017), with the possibility of using isotopic internal calibration to reduce the need for batch-type analysis (Couchman *et al.*, 2013). These analytical advancements could enable faster turn-around-times of the results at lower cost, which in turn could improve the utility of TDM in optimising antipsychotic therapy.

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Appendices

Appendix A. Ethics letter of approval and protocol for the collection of clinical samples

Included herein:

- Approval notice for ethics (2 pages)
- Patient information and consent form (4 pages)
- Sample collection protocol (6 pages)

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Approval Notice

New Application

07-Feb-2014
Beyer, Chad C

Ethics Reference #: S13/11/227

Title: **Assessing the measurement of antipsychotics, particularly clozapine, in oral fluids using the Greiner bio-one buffered collection system in comparison with the Cozart collection system and normal plasma measurements.**

DearMr Chad Beyer,

The **New Application** received on **11-Nov-2013**, was reviewed by members of **Health Research Ethics Committee 1** via Minimal Risk Review procedures on **03-Feb-2014** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **07-Feb-2014 -07-Feb-2015**

Please remember to use your **protocol number** (**S13/11/227**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required **BEFORE** approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219389156.

Included Documents:
Consent Form
General Checklist
CV - Fisher

Declaration - Flanagan
Full protocol
Synopsis
Declaration - Beyer
Application Form
Declaration - Seedat
CV - Seedat
CV - Flanagan
Declaration - Fisher
CV - Beyer

Sincerely,

Franklin Weber
HREC Coordinator
Health Research Ethics Committee 1

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:
Assessing the measurement of antipsychotics, particularly clozapine, in oral fluids using the Greiner bio-one buffered collection system in comparison with the Oral-Eze Oral-Fluid collection system and normal plasma measurements.

REFERENCE NUMBER:

INVESTIGATOR: Chad Beyer

PRINCIPAL INVESTIGATOR: Prof S Seedat

ADDRESS: Room 2004, Department of Psychiatry, 2nd Floor, Faculty of Medicine and Health Sciences, Francie van Zijl Dive, Parow, 7505

CONTACT NUMBER: (021) 9489227, 0827848148

You are being invited to take part in a research project.
Please take some time to read the information presented here, which will explain the details of this project. Please do not hesitate to ask the study staff or doctor any questions about any part of this project which you do not fully understand.
It is very important that you are fully satisfied that you clearly understand what this research is about and how you could be involved.
Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not negatively affect you in any way whatsoever.
You are also free to stop being involved with this study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee (HREC) at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?
The purpose of this study, which is being conducted at Tygerberg and Stikland Hospitals, is to see if using a buffered solution to collect saliva is better than a normal saliva sample, instead of blood, to measure the amount of medication that you are taking in your body.
A buffer is a type of fluid that makes the acid in your mouth equal to a level we can measure. It is not harmful to you in any way.
The medication we are testing is known as an anti-psychotic. In order to do this, we will be collecting both blood and two saliva samples from 100 people on antipsychotics. If we are succesful, this study may mean that future patients will be able to give saliva instead of blood samples, which will be more comfortable for all patients, especially children.
It may also allow us to do more research on these medications in the future without having to take a blood sample.

Why have you been invited to participate?
As a person who is being treated with an anti-psychotic medication, you will be able to assist us with this research.

What will your responsibilities be?

- Provide us with some basic, non-personal information,
- Allow us to draw around a tablespoon of blood
- Spit into a container for us
- Hold a coloured liquid (the buffer) in your mouth for two minutes before spitting it into another container for us

Please inform us if you are allergic to aspirin, benzoic acid or are asthmatic.

Will you benefit from taking part in this research?
You will not directly benefit from taking part. However, you may be helping future patients on these medications, especially children, as it may mean that we won't have to draw blood to measure drug levels anymore. It will also make it easier for us to study how the anti-psychotic levels change in the body over time more which will make these drugs safer for everyone taking them.

Are there in risks involved in your taking part in this research?

- If you are asthmatic, or allergic to aspirin or benzoic acid the buffer (coloured fluid) can trigger an episode
- You may experience some pain and discomfort from the drawing of the blood sample
- There are no other risks to participating in this study.

If you do not agree to take part, what alternatives do you have?
This study is entirely voluntary and there are no penalties at all for not wanting to participate.

Who will have access to your medical records?
Nobody will have access to your medical records, other than the small amount of information we obtain from you on a form which doesn't contain any of your personal details, and cannot be traced back to you.

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?
Injury is extremely unlikely, but if you are hurt in someway you will receive appropriate care from the hospital in which you are present.

Will you be paid to take part in this study and are there any costs involved?
No, you will not be paid to take part in the study and you will not be responsible for any other costs.

Is there any thing else that you should know or do?

- You can contact Mr Chad Beyer at 082 458 2707 or Professor S Seedat at 021 938 9374 if you have any further queries or encounter any problems.
- You can contact the **Health Research Ethics Committee** at 021 938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ..... agree to take part in a research study entitled: **Assessing the measurement of antipsychotics, particularly clozapine, in oral fluids using the Greiner bio-one buffered collection system in comparison with the Oral-Eze Oral-Fluid collection system and normal plasma measurements.**

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable and which I understand.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ..... on (*date*) ..... 20.....

....

Signature of participant **Signature of witness**

Declaration by investigator

I, Chad Beyer declare that:

- I explained the information in this document to .....
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ..... on (*date*) ..... 20.....

....

Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ..... declare that:

- I assisted the investigator (*name*) ..... to explain the information in this document to (*name of participant*) ..... using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ..... on (*date*) .....

....

Signature of interpreter **Signature of witness**

Research Protocol

Research Protocol Summary:

Assessing the measurement of antipsychotics, particularly clozapine, in oral fluids using the Greiner bio-one buffered collection system in comparison with the Oral-Eze Oral-Fluid collection system and normal plasma measurements.

Investigators: Mr Chad Beyer, Dr Gareth Nortje , Ms Irene Mbanga

Local supervisor and Principal Investiagator: Professor Soraya Seedat

International collaborators: Miss Danielle Fisher (Department of Clinical Biochemistry at Kings College Hospital NHS trust, London); Professor Robert Flanagan (Department of Toxicology, King's College Hospital, London); Dr Fiona Gaughran (NHS Foundation trust, South London and Maudsley)

Context of study and literature review

This study is a sub-study to a parent study being conducted at Kings College London on the need to implement accurate non-invasive measures of drug concentrations so as to ensure they remain within therapeutic levels. Plasma TDM has been well established for some atypical antipsychotics including clozapine, olanzapine, and amisulpride, and TDM is recommended especially when assessing adherence and in patients undergoing clozapine dose titration (Hiemke *et al.*, 2011).

A barrier to therapeutic drug monitoring is the problem of collecting blood samples; this can be due to problems with patient compliance or with anatomical concerns such as collapsed veins, or difficulty in locating a vein. Furthermore, the nature of the patients who are prescribed antipsychotics means that they are often difficult to obtain samples from as they can be disinclined to submit to routine plasma monitoring. Our study aims to investigate whether we can ascertain if the use of saliva as an alternative to plasma as alternative matrices will be able to provide accurate, in terms of adherence to medication and therapeutic levels, so as to reduce the need for blood sampling (Fisher et al., 2013; Patsalos and Berry, 2013).

Existing research into this topic is limited, and for the most part restricted to research on rispiridone. At least one study found a strong correlation between blood and salivary levels of risperidone, and highlighted the relevance of this finding to pharmacodynamic research and therapeutic drug monitoring (Aman et al., 2007).

The most common method for drugs to enter saliva is by way of passive diffusion, which translates to the fact that drug concentrations in oral fluid are

affected by the oral fluid's pH and the drug's pKa, in addition to other parameters (Aps and Martens, 2005). Previous work done by this institution found that oral fluid collected by the drool method in 90 patients showed a relatively poor correlation (R^2 0.3-0.7) between the plasma and oral fluid concentrations measured (Fisher et al., 2013). This work did, however, show potential for oral fluid measurement for analysis of drugs of interest, although the inter-patient variation in the plasma:oral fluid ratio was large. One proposed explanation for this was the inter-patient variation in salivary pH.

Comparisons of various oral fluid collection devices showed that, in vitro, those that contained a buffer improved the recovery and stability of the analytes measured. (Langel *et al.*, 2008). One type of buffered collection system is that produced by Greiner Bio One. This consists of four component parts: (1) a mouth wash that the patient uses immediately prior to collection, (2) a 4 mL buffer containing a food dye that is held in the mouth without swallowing, (3) a beaker into which the buffer solution is spat after 2 minutes, and (4) two tubes that are used to securely collect the sample from the beaker to be sent for analysis (Greiner Bio One, 2011). Sodium citrate is used to buffer the solution, and the degree of dilution of the dye (tartrazine) is used to give indication of the amount of oral fluid collected. This would theoretically translate to all samples being collected and maintained under the same system, accounting for and making irrelevant the inter-individual variation in saliva pH and flow-rate. The latter is compensated for by a known amount of dye ion the collection fluid being used. An issue with this is there are reported allergies to this dye amongst those sensitive to aspirin or benzoic acid; or amongst asthmatics.

In South Africa, antipsychotic drugs are widely prescribed in both children and adolescents for the treatment of several conditions, including schizophrenia and bipolar disorder. Invariably, issues such as adherence are of particular concern in this population group. Validating salivary anti-psychotic measurement could eventually lead to better informed dosing practices in children and adolescents, and provide a convenient method for monitoring of adherence and optimising dosage. The non-invasive nature of salivary sampling furthermore increases the possibilities for serial sampling and in-depth study of salivary drug concentrations over time.

Aims and objectives

The main objective of this study is to ascertain the accuracy, feasibility and applicability of measuring clozapine and other antipsychotics using a buffered oral collection medium. Our hypothesis is that when salivary pH and flow rate are accounted for oral fluid will be an accurate, feasible and applicable predictor of the plasma concentration of the drugs studied.

Methods

This study is laboratory-based, and involves the collection of paired blood and two saliva samples from approximately 150 participants. The exact steps of the process are outlined below:

1. Ethics will be obtained from Stellenbosch University Health Research Ethics Committee. (This has been obtained - #S13/11/227.)
2. Permission will be sought from the relevant hospital authorities (Tygerberg, Stikland and Lentegeur Hospitals).
3. Registrars in psychiatry will be asked to refer potentially eligible patients.
4. GN or IM will visit patients the day before sampling to assess eligibility and obtain written informed consent. IB or GN will explain the purpose of the study and the process of sample collection.
5. IM and GN will collect the samples in the morning from both inpatients and outpatients at Tygerberg, Lentegeur and Stikland Hospitals.
6. Adult patients (18 years and older) on male and female psychiatric wards (Tygerberg and Stikland hospitals) who are close to discharge are eligible.
7. Sample collection will involve the drawing of approximately 5 mL of venous blood into a BD EDTA (purple top) tube, the use of a fluid collector with a collection pad for the Oral-Eze sample and a salivary sample (more accurately an oral fluid sample) as per the Greiner Bio-one collection method. The Greiner Bio-one method is as follows: the patient uses a provided a mouth wash immediately prior to collection, then holds a 4 mL buffer containing a food dye in the mouth without swallowing, then spits the buffer into a beaker after 2 minutes, and then using two tubes the sample is securely collected from the beaker to be sent for analysis
8. Relevant details will be recorded, preserving patient anonymity using unique study reference numbers. Details include:
    a. Date and time of sample collection
    b. The drug and dosage currently being used by the patient
    c. The last time when the drug was taken

    This information will be recorded on a special form, which is included in this protocol. The collection will be anonymous, and the form will be linked to the samples by a study identification number.
9. IM or GN will take samples to the NHLS laboratory each day for pre-processing and storage (freezing).
10. Once approximately 150 samples have been collected, the samples will be sent to King's College Hospital Toxicology Lab in London for analysis. Permission will be sought from the department of health for the transportation of the samples.
11. The Toxicology Unit at King's College Hospital, London will analyse the samples using Liquid Chromatography-Mass Spectrometry.
12. Following the outcome of the laboratory analysis the results will be statistically analysed using SPSS version 20. Log transformation will be applied if the data are not normally distributed. Mean, SD and range will be generated for plasma and saliva antipsychotic levels. Both salivary levels will be compared with plasma levels by using the Pearson's correlation coefficient.

Study Inclusion criteria

1. The patient must be taking an atypical antipsychotic.
2. The morning dose for the antipsychotic must have been omitted.
3. The patient must not be a known illicit drug user.
4. The patient must give informed consent for participation in the study. The sample consent form, which will also be verbally explained to the participant, is included in this protocol.
5. The patient must be over eighteen years of age.

Study Exclusion criteria

1. Patients less than 18 years of age.
2. Patients who are unable to understand and comply with the informed consent procedure (i.e. by virtue of disorganised behaviour, thought disorder or marked cognitive impairment).
3. Patients who are on conventional ('typical' antipsychotic drugs).
4. Patients who have not omitted their morning dose of the antipsychotic.
5. Patients who have a known sensitivity to sensitive to aspirin or benzoic acid;
6. Patients who have been diagnosed as having, or are suspected to have, asthma.

Data management

Limited data will be collected with each sample, consisting of the patient's age, gender, suburb of residence, diagnosis, current medication and dosage, and timing of last dose. A unique and anonymous reference number will be paired with each sample, and separately paired with the patient's name. Thus the clinical data will be completely anonymous after collection. The name-reference number pairing data will only be accessed by IM and GN at the time of sampling, and be kept securely and confidentially in an encrypted database by GN.

Time plan and logistics

Our goal is to complete sampling by the end of December 2014. The samples will then be sent to London, where another two months will be allowed for processing and analysis. A further two months will be dedicated to the statistical analysis and writing up of the results.

The local investigators, IM and GN, along with supervisor, Professor Seedat, will be responsible for all aspects related to sample collection and transportation. Professor Flanagan and Dr Gaughra of King's College Hospital, London, will be responsible for analysing the samples.
Data will be analysed by the local investigators, CB and GN, with assistance from experienced statisticians within the Faculty of Medicine and Health Sciences of Stellenbosch University. The local investigators will then write an article in conjunction with all the team members. This article will then be submitted for publication in an appropriate journal.

Ethical aspects

The main concern raised by the study is the discomfort it may cause to patients, with no direct benefit to their own care. In this light, the process of informed consent must be fully adhered to. This in and of itself is a problem in the context of patients using antipsychotics, as their ability to consent to such involvement is at times unclear. In order to minimize this problem, informed consent will only be obtained in conjunction with the practitioner primarily responsible for the patient's care, who will then determine whether or not he/she feels the patient is sufficiently competent to make such a decision. Wherever possible, the blood sample collection will be made to coincide with occasions where venepucture would be indicated for medical reasons.

Although the information collected will be of a non-sensitive nature, the blood samples will be anonymised using the aforementioned numerical referencing system, and once this has been done the samples will only bear a link to the data form with the same number. The patient's name will not be included on this form. In this way, no sample can be linked back to a specific patient. An example of the data capture sheet as well as the informed consent form is included with this protocol.

Budget

Sample collection costs will be covered by SARChI research fund allocated to Prof. Soraya Seedat. These amount to R1500.

The Greiner Bio-One and Oral-Eze salivary collection kits are supplied by King's College Toxicology Lab, London, free of charge.

Pre-processing and storage at the Tygerberg NHLS laboratory costs R7500 for 150 samples.

Couriering the samples to the Toxicology Unit at King's College Hospital in London is paid for by the collaborators at King's College Hospital.

Local transport and sundry costs will be R1000.

Total budget: R10,000

Strengths and limitations

The main strength of this study is that it allows for pooling of samples from two different institutions for analyses. Furthermore this study allows comparison between buffered and unbuffered oral fluid, the former accounting for what is postulated to be the main confounding variable in the large inter-individual differences experienced in previous studies. In accounting for this variable this study will likely generate significant data.

An initial limitation to this study will be due to the focus on clozapine as the antipsychotic of choice in terms of measurement. However, this limitation will be overcome when the study is more generalised, including other atypical antipsychotics.

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Appendix B. Within- and between-batch IQC results

Included herein:

- Within- and between-batch IQC replicate results from which to calculate the inaccuracy and imprecision for the GBO device method (Tables A-1 and A-2)
- Within- and between-batch IQC replicate results from which to calculate the inaccuracy and imprecision for the Oral-Eze device method (Tables A-3 and A-4)

Table A-1: Individual within-batch IQC results for GBO system method.

Nominal [analyte] (µg/L)		Replicate result (µg/L)				
		1	**2**	**3**	**4**	**5**
Amisulpride	6	7	7	6	7	7
	75	65	74	69	69	72
	300	265	279	290	289	289
Aripiprazole	6	5	3	4	3	4
	75	32	34	34	34	34
	300	221	215	222	217	214
Dehydroaripiprazole	6	4	2	3	3	3
	75	31	32	32	33	32
	300	236	217	220	221	221
Clozapine	60	52	50	49	51	52
	300	281	277	271	275	272
	900	860	907	886	834	843
Norclozapine	60	53	48	47	50	51
	300	261	277	271	276	277
	900	824	834	836	845	841
Fluoxetine	6	7	5	5	6	7
	75	66	86	70	80	78
	300	271	334	333	279	238
Norfluoxetine	6	7	5	5	6	7
	75	82	85	77	75	83
Olanzapine	3	3	3	3	3	3
	30	25	26	26	25	26
	90	81	76	76	82	80
Quetiapine	6	7	6	6	6	7
	75	68	67	65	66	66
	300	277	281	269	276	284
N-Desalkylquetiapine	6	8	6	6	7	7
	75	73	66	64	67	68
	300	278	272	289	254	261
O-Desalkylquetiapine	3	3	3	3	3	3
	30	32	30	29	30	30
	90	93	92	91	89	91
7-Hydroxyquetiapine	3	3	3	3	3	3
	30	29	29	29	31	30
	90	92	88	90	84	90
Risperidone	3	3	3	3	3	3
	30	29	28	29	29	30
	90	87	87	90	87	88
9-Hydroxyrisperidone	3	4	3	3	3	3
	30	30	29	30	28	28
	90	87	85	93	92	85
Sulpiride	6	8	6	7	6	6
	75	84	89	84	82	87
	300	338	323	317	290	320

Table A-2: Individual between-batch IQC results for GBO system method.

Nominal [analyte] (µg/L)		Replicate result (µg/L)			
		1	**2**	**3**	**4**
Amisulpride	6	7	7	7	7
	75	67	70	72	73
	300	278	282	282	293
Aripiprazole	6	2	4	4	3
	75	22	34	30	30
	300	219	218	225	191
Dehydroaripiprazole	6	2	3	3	3
	75	21	32	30	33
	300	215	223	238	211
Clozapine	60	51	51	49	48
	300	322	275	275	267
	900	872	866	843	823
Norclozapine	60	53	50	49	49
	300	287	272	271	261
	900	895	836	845	825
Fluoxetine	6	6	6	6	5
	75	88	78	69	65
	300	291	289	294	285
Norfluoxetine	6	8	6	7	6
	75	68	80	89	79
Olanzapine	3	3	3	3	3
	30	28	26	25	24
	90	83	79	78	74
Quetiapine	6	6	7	6	6
	75	67	66	67	65
	300	283	278	271	273
N-Desalkylquetiapine	6	6	7	6	6
	75	63	68	68	69
	300	279	271	274	284
O-Desalkylquetiapine	3	3	3	3	3
	30	31	30	30	30
	90	88	91	90	91
7-Hydroxyquetiapine	3	3	3	3	3
	30	29	29	29	28
	90	110	89	84	85
Risperidone	3	3	3	3	3
	30	27	29	28	29
	90	83	88	87	87
9-Hydroxyrisperidone	3	3	3	3	3
	30	32	29	28	29
	90	97	88	86	87
Sulpiride	6	6	6	7	7
	75	70	93	85	93
	300	336	317	316	335

Table A-3: Individual within-batch IQC results for Oral-Eze system method.

Nominal [analyte] (µg/L)		Replicate result (µg/L)				
		1	**2**	**3**	**4**	**5**
Amisulpride	6	7	6	7	7	7
	75	75	72	72	70	74
	300	309	304	293	301	288
Aripiprazole	6	11	11	11	11	12
	75	82	90	85	84	86
	300	362	321	341	350	342
Dehydroaripiprazole	6	12	12	12	12	12
	75	97	107	104	105	102
	300	430	377	412	397	407
Clozapine	60	68	68	71	70	68
	300	299	283	301	281	288
	900	914	935	907	943	924
Norclozapine	60	69	67	67	66	70
	300	271	275	258	276	286
	900	883	882	905	945	877
Fluoxetine	6	5	8	7	7	6
	75	64	84	60	67	64
	300	326	312	277	279	343
Norfluoxetine	6	9	9	9	6	9
	75	87	74	67	79	77
Olanzapine	3	4	4	4	4	4
	30	32	34	32	33	31
	90	96	98	98	102	102
Quetiapine	6	6	5	6	6	6
	75	56	56	55	53	53
	300	221	233	221	228	221
N-Desalkylquetiapine	6	9	8	7	8	8
	75	79	77	80	80	82
	300	363	339	319	335	328
O-Desalkylquetiapine	3	3	3	3	3	3
	30	28	30	30	28	29
	90	85	86	84	86	82
7-Hydroxyquetiapine	3	3	2	2	3	3
	30	25	25	22	24	25
	90	75	69	65	74	77
Risperidone	3	4	3	4	4	4
	30	32	32	31	32	31
	90	93	96	99	104	94
9-Hydroxyrisperidone	3	4	3	3	3	3
	30	32	32	30	32	34
	90	105	93	95	97	102
Sulpiride	6	8	5	7	7	5
	75	98	88	79	90	79
	300	342	393	318	352	329

Table A-4: Individual between-batch IQC results for Oral-Eze system method.

Nominal [analyte] (µg/L)		Replicate result (µg/L)			
		1	**2**	**3**	**4**
Amisulpride	6	7	7	7	7
	75	75	76	73	73
	300	303	314	299	303
Aripiprazole	6	11	12	11	12
	75	89	94	85	93
	300	350	368	343	367
Dehydroaripiprazole	6	11	12	12	12
	75	102	111	103	110
	300	392	430	404	417
Clozapine	60	71	73	69	71
	300	295	295	290	280
	900	926	962	925	927
Norclozapine	60	70	70	68	58
	300	281	279	273	222
	900	895	895	898	698
Fluoxetine	6	7	6	7	7
	75	87	85	69	66
	300	305	335	307	270
Norfluoxetine	6	9	10	9	8
	75	79	74	77	60
Olanzapine	3	5	5	4	8
	30	31	35	33	36
	90	95	103	99	97
Quetiapine	6	7	6	6	6
	75	58	54	55	54
	300	239	224	225	226
N-Desalkylquetiapine	6	9	9	8	8
	75	87	91	80	82
	300	353	375	337	358
O-Desalkylquetiapine	3	3	3	3	3
	30	27	28	29	23
	90	80	84	85	75
7-Hydroxyquetiapine	3	2	3	3	3
	30	26	24	24	24
	90	77	78	72	69
Risperidone	3	4	4	4	4
	30	32	33	31	32
	90	95	98	97	96
9-Hydroxyrisperidone	3	4	4	3	4
	30	33	33	32	34
	90	97	106	98	115
Sulpiride	6	8	7	6	6
	75	82	95	87	97
	300	319	369	347	333

Appendix C. Publications

Included herein are complete copies of relevant publications produced prior-to or during this project:

- Fisher D.S., Beyer C., van Schalkwyk G., Seedat S., and Flanagan R.J. (2017). Measurement of Clozapine, Norclozapine, and Amisulpride in Plasma and in Oral Fluid Obtained Using 2 Different Sampling Systems. *Ther Drug Monit*. 39, 109-117.
- Fisher D.S., van Schalkwyk G.I., Seedat S., Curran S.R., and Flanagan R.J. (2013A). Plasma, oral fluid, and whole-blood distribution of antipsychotics and metabolites in clinical samples. *Ther Drug Monit*. 35, 345-351.
- Fisher D.S., Partridge S.J., Handley S.A., Couchman L., Morgan P.E., and Flanagan R.J. (2013B). LC-MS/MS of some atypical antipsychotics in human plasma, serum, oral fluid and haemolysed whole blood. *Forensic Sci Int*. 229, 145-150.
- Fisher D.S., Handley S.A., Flanagan R.J., and Taylor D.M. (2012A). Plasma concentrations of quetiapine, N-desalkylquetiapine, O-desalkylquetiapine, 7-hydroxyquetiapine, and quetiapine sulfoxide in relation to quetiapine dose, formulation, and other factors. *Ther Drug Monit*. 34, 415-421.

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